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Critical and Independent Roles of the P/CAF Acetyltransferase in ARF-p53 Signaling: A Dissertation

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CRITICAL AND INDEPENDENT ROLES OF THE P/CAF ACETYLTRANSFERASE IN p53-ARF SIGNALING

A Dissertation Presented

By

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I'd also like to thank my wife Angie for being so supportive during this process – I'm very grateful for her patience during the stressful times that graduate work sometimes entails, and I wish her the best of luck in her pursuit of a degree. It hasn't always been the easy, but her optimism has helped me see all the things I'll miss so much about this institution and the people who make it what it is. Thanks to my family as well, for stimulating and supporting my interest in science throughout my life, for challenging me to excel in everything I do, and for always being supportive no matter the situation.

Lastly, thanks to the great many friends I've made in my six years here, and the support, good times, and many laughs they've provided me with; the helpful scientific and career discussions we've all had over the years; and, most importantly, the stories and experiences that I'll take with me for the rest of my life, wherever I go.

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Preface

All the research performed in this thesis was performed by me in the Androphy and Grossman labs unless specifically noted below. The research presented in Chapter II was performed under the resources and guidance of the Androphy and Grossman labs. I was responsible for all the work shown in Chapter II, which was performed exclusively in the Grossman lab with the exception of the transfections and western blots in Figure 2.8, which were performed by Dr. Pedja Sekaric, PhD, a former post-doctoral associate of the Androphy lab. The work presented in Chapter III was performed in the Androphy lab. The western blots shown in Figure 3.2 were performed by Dr. Vladimir Shamanin, PhD, a former research assistant professor of the Androphy lab. I was responsible for the remaining work in Chapter 3. I also performed the experiments presented in Appendix I in the Androphy lab.

Abstract

For 30 years, the tumor suppressor p53 has been a subject of intense research in nearly every discipline of scientific inquiry. While numerous surprising roles for p53 in health and disease are uncovered each year, the central role of its activation in preventing neoplastic transformation has been and will remain at the forefront of p53 research as investigators work to address an unexpectedly complex question – precisely how does p53 integrate upstream stress signals to coordinate activation of its target genes in response to stress?

One manner in which to address this question is at the level of transcription initiation – after upstream signals converge on p53 and produce a number of pools of post-transcriptionally modified p53, how exactly are specific target promoters activated in such a sensitive, context-specific manner? The work presented herein aims to address the role of histone acetylation at the p21 promoter – a critical mediator of G1/S arrest - by the P/CAF acetyltransferase in response to a variety of p53-activating stresses. We show that depletion of P/CAF strongly inhibits p21 expression in response to a variety of stresses, despite normal stabilization of p53 and recruitment to target promoters. This defect in p21 expression correlates closely with abrogation of stress-induced cell-cycle arrest. Strikingly, a p53 allele lacking putative P/CAF acetylation sites was still able to direct p21 expression, which was still dependent upon P/CAF. We show further that histone acetylation at H3K14 at the p21 promoter following stress is dependent upon P/CAF. Rescue of p21 expression with wild-type P/CAF or a ΔHAT point mutant

indicates that P/CAF requires an intact HAT domain, suggesting that histone acetylation at H3K14 is catalyzed by P/CAF HAT activity, not the molecular bridging of a heterologous HAT by P/CAF. Furthermore, RNA polymerase II (RNAP II) was present at the p21 proximal promoter under all basal and stress conditions, but elongation of RNAP II after stress required the presence of P/CAF. These data indicate that H3K14 acetylation by P/CAF closely correlates with the activation status of the p21 promoter, and may be necessary for activation of a larger subset of p53-responsive promoters.

In addition to its critical role in p21 expression, we noted that p53 stabilization and cell-cycle arrest in response to p14ARF, but not other p53-stabilizing stresses, were also dependent on P/CAF. Cell-cycle arrest induced by p16INK4A was intact after P/CAF ablation, indicating a role for P/CAF in cell-cycle arrest specific to p14ARF-p53 signaling. Basal MDM2 levels were unaffected by P/CAF knockdown, as were p53-MDM2 and ARF-MDM2 complexes. A preliminary analysis of MDM2 localization was inconclusive, due to vastly different quantities of MDM2 in different conditions making analysis of subcellular localization difficult; however, the role of P/CAF in the relocalization of MDM2 to the nucleolus by p14ARF could potentially explain the defect in p53 stabilization, and should be explored further.

These observations, underscored by recent reports that P/CAF undergoes loss of heterozygosity in several tumor types, suggest that P/CAF plays a critical role in p53-mediated cell-cycle arrest through multiple, independent mechanisms. Further study should clarify whether P/CAF is lost in tumors maintaining wild-type p53, and whether its reintroduction into these tumors confers any potential therapeutic benefit.

Chapter I

Introduction

Principles of Tumorigenesis

Human cancer is a class of devastating, genetically heterogeneous diseases which, unlike many illnesses, spares no demographic; people of all social, economic, and ethnic backgrounds are afflicted. At its core, cancer is a disease characterized by unrestrained proliferation of a group of cells in the body. Cancer can arise in nearly any tissue type in the body and, through its ability to interfere with normal tissue function, can wreak havoc on normal human physiology; in 2008 alone, about 7.6 million deaths were caused by cancer – 13% of all worldwide deaths (Jemal et al., 2011). Death caused worldwide by malignancies is currently overshadowed only by cardiovascular and infectious disease (Jemal et al., 2011).

Cancers arise from a complex interaction of a multitude of genetic and environmental factors. Many well-documented occupational and recreational hazards exist, the most publicized in the developing world being asbestos and cigarette smoke exposure, respectively. Many cancer-causing infectious diseases also exist which promote cancer directly through encoded oncoproteins, or indirectly by creating an environment suitable for oncogenic mutations to arise. While many causative genetic links to cancer exist, even the most well-studied hereditary mutations usually account for not more than a fraction of total occurrences; in the absence of aggressive screening, mutation of the APC tumor suppressor results in highly penetrant colon cancer still accounting for only approximately 20-30% of cases (Rustgi, 1993). Similarly, mutations in the highly

publicized breast cancer-associated genes BRCA1 and BRCA2 account for an estimated 3-5% of breast cancers (Brody and Biesecker, 1998), supporting the idea that cancer is a family of complex, multifactorial diseases.

Incidence of most cancers accelerates sharply with age (Rustgi, 1993) – an observation consistent with a stochastic, multi-step process in which cumulative changes take place over time to yield a malignancy (Weinberg, 1998). Although there is widespread variability in the latency of onset of various cancers, evidence suggests that four to six rate-limiting (Armitage and Doll, 1957; Fearon and Vogelstein, 1990) events must occur in a single cell lineage; this explains in part why inherited cancer-associated genetic mutations result in disease with highly variable penetrance (Hanahan and Weinberg, 2000). These four to six rate-limiting steps translate to the perturbation of a corresponding number of specific cellular functions.

As mentioned above, unchecked replication of a population of cells is a necessary characteristic of all cancerous growths; however, this trait alone is insufficient for a tumor to be classified as cancerous; in addition to unrestricted growth potential, tumors must acquire several additional capabilities to become malignant and, in most cases, to significantly interfere with normal physiological functions. In addition, normal cells can also acquire traits such as genomic instability which increase the likelihood of subsequent tumorigenic events occurring. While the temporal order of acquisition of these additional required characteristics is still under intense investigation (and, given the broad heterogeneity of tissue types and nucleating genetic or environmental events, highly complex), broad agreement exists regarding the changes required to give rise to a

malignant phenotype. Broadly, these properties work together to enhance the survival of tumor cells through a variety of mechanisms.

Metazoans have evolved a complex, highly-regulated system of checkpoints to ensure fidelity of DNA replication, controlled proliferation, and proper cell identity. Malignant tumors represent a rare evasion of these control mechanisms, which include intrinsic limitation of proliferation through telomere attrition; resistance to intrinsic and extrinsic growth-suppressive or apoptotic stimuli; sustained growth signaling; and the characteristics that underly a true malignant phenotype – the generation and maintenance of new vasculature, and the activation of signaling pathways which allow for invasion of neighboring tissue and metastasis. These acquired traits of cancers are referred to as the 'hallmarks' of cancer, but ongoing research continues to uncover additional acquired traits of cancers (Hanahan and Weinberg, 2011).

In addition to these well-supported hallmarks of cancer, genomic instability also contributes to cancer onset by shortening tumor latency. Genomic instability can be broadly defined as an increased tendency of the genome of a cell or clonal population to acquire mutations during each DNA replication cycle. Ongoing debate still rages over the impact of genomic instability in the onset of cancer; while some groups argue that genomic instability is necessary and selected for early in tumorigenesis (Loeb, 1991), other evidence suggests that an increased rate of cell proliferation is sufficient to allow the accumulation of deleterious mutations (Tomlinson and Bodmer, 1999). There are indeed several examples of diseases which promote rapid and sustained cell proliferation and are associated with increased cancer risk, despite having no direct impact on genomic

stability; in the liver, hepatocellular carcinoma is a common complication of cirrhosis brought on by alcoholism, hepatitis B, or hepatitis C, wherein the liver is under constant pressure to replace large populations of dead or dying hepatocytes. It is likely that both the rate of cell proliferation and the stability of the genome independently contribute to cancer risk, and therefore that intrinsic mechanisms impacting these variables, in addition to the aforementioned hallmarks of cancer, are also important risk determinants. The study of cancer biology is in essence the study of the genetic, epigenetic, and environmental factors that modulate these processes.

The genes and gene products acted upon by these influences whose alteration impacts cancer risks are generally classified as tumor suppressors, those genes whose loss of function or expression increase cancer risks through perturbation of normal function; or oncogenes, those genes which increase cancer risk through gain of function or increased expression. Both tumor suppressors and oncogenes function in a multitude of cellular pathways not easily categorized but that, when perturbed, can confer a selective advantage to the affected cell.

p53: Oncogene or Tumor Suppressor?

For many years, a great deal of research was focused on oncogenic viruses and the methods by which these viruses hijack normal cellular processes to promote cancer. It quickly became clear that many viruses promote cancer through the expression of oncogenes encoded by their genomes which closely resembled host protooncogenes; these genes were found to be ‘hijacked’ from the host genome, as they often conferred a

selective advantage to the viral particles (Stehelin et al., 1976). Despite this discovery, the viral oncoproteins often bore little resemblance to cellular oncogenes, raising the question of how these proteins carry out their oncogenic functions (Stehelin et al., 1976). One such transforming protein encoded by simian-virus 40 (SV40), known as large-T antigen, was soon found to interact strongly with a host-encoded 53kD protein (Lane and Crawford, 1979).

Initial Characterization as a Tumor Antigen

At the time p53 was identified as a binding partner of Large-T, cellular interactors of viral oncogenes were themselves bearers of oncogenic activity, and p53 was believed to be no exception. Further evidence continued to make the case for p53 as a oncogene or 'tumor-antigen'; in normal cells, p53 was present at low or undetectable levels, while transformed cells exhibited excessively high levels of p53 (Rotter et al., 1980). This trend held true in a variety of experimental settings, continuing to lend support to the idea that high p53 levels correlated with the presence of viral oncogenes - causative agents of cellular transformation.

After evidence continued to mount in support of the role of p53 as an oncogene, several groups undertook the task of cloning the p53 cDNA to more thoroughly study this tumor-associated protein's properties. Several labs generated mouse and human cDNAs from transformed cells with high levels of p53, which facilitated easier cloning (Harlow et al., 1985; Oren and Levine, 1983; Wolf et al., 1985). The cDNA product was used in classical cooperation assays, in which two factors are introduced into cultured cells and

assayed for their ability to yield a transformed phenotype wherein cells exhibit tumorigenic properties of contact-independent growth, lack of contact inhibition, and the ability to form tumors in nude mice. As many expected and among several other lines of supportive evidence, the p53 cDNA cooperated with Ras to transform cultured cells (Eliyahu et al., 1984; Parada et al., 1984). It seemed unquestionable that this 53kD binding partner of SV40 Large-T was itself contributing to tumorigenesis.

Wild-type p53 as a Tumor Suppressor

In spite of the mountain of evidence characterizing p53 as an oncogene, lines of evidence began to emerge that questioned the simplicity of this conclusion. As more labs cloned p53 from different sources, it became clear that not all p53 clones exhibited transforming activity and, further, that p53 was inactivated in mouse cells transformed by several oncogenic viruses (David et al., 1988; Wolf and Rotter, 1984). Further investigation through direct sequence analysis indicated that the first clones isolated and analyzed exhibited sequence variations, raising the possibility that p53 isolated from these tumor cells was in fact mutated (Levine and Oren, 2009). When p53 was finally isolated from normal murine cells, this was shown unequivocally to be the case (Finlay et al., 1988). Characterization of the wild-type p53 protein then began, starting with the analysis of p53 loci in various tumors. Loss of heterozygosity, a hallmark of tumor suppressors, was found to occur in colorectal tumors through mutation or allelic loss (Baker et al., 1989). Additionally, in stark contrast to the potent transforming activity of early p53 clones with activated Ras, wild-type p53 suppressed the transforming activity

of known cooperating oncogenes (Eliyahu et al., 1989). Finally cementing the status of wild-type p53 as a tumor suppressor were the subsequent observations that Li-Fraumeni syndrome, characterized by early-onset cancer, is caused by p53 mutations (Malkin et al., 1990), and that p53-knockout mice exhibit high susceptibility to tumors (Donehower et al., 1992). With p53 now defined as a classical tumor suppressor, attention would immediately turn to how these effects are exerted.

p53 is a Sequence-specific Transcription Factor

In the many years since the final designation of p53 as a tumor suppressor, numerous cellular activities have been described for p53. Broadly, p53 serves to integrate various upstream stress sensors and responses with an appropriate cellular outcome. The first major functions to be attributed to p53 were potent DNA-binding (Kern et al., 1991) and transcriptional activation activities (Raycroft et al., 1990). Strikingly, these activities were absent in most of the tumor-derived p53 mutants, strongly suggesting that these activities contribute significantly to p53-mediated tumor suppression.

Tumor suppression by p53 relies primarily upon its ability to transcriptionally activate target genes. About 50% of all cancers express mutant p53, and nearly all of these p53 mutants are now known to harbor missense mutations in the DNA-binding domain, rendering it incapable of either positively or negatively regulating transcription through DNA binding. Mouse knock-in models further support the role of p53 transcriptional activities in tumor suppression, as do several correlative studies of Li-Fraumeni patients, wherein transcriptional capabilities of inherited p53 mutants

correlated with clinical manifestation of resulting tumors (Monti et al., 2007).

The importance of p53 transcriptional activity in tumor suppression is unquestionable, and will be described in further detail below. However, it is important to note that, although most attention has focused on p53 as a transcriptional activator, p53 also performs several important transcription-independent functions, including but not limited to transcriptional repression (Ho and Benchimol, 2003), fine-tuning DNA damage repair by homologous recombination (Bertrand et al., 2004), and involvement in mitochondrial outer membrane permeabilization critical to induction of apoptosis (Vaseva and Moll, 2009).

Control of p53 Activity: the MDM2-p53 Feedback Loop

The ability of a single protein to eliminate a cell from the replicative pool necessitates that its activities be kept checked under basal conditions. In contrast to the massive overexpression of mutant p53 in many tumors, wild-type p53 is kept at very low basal levels in most unstressed cells. This is achieved through the activity of the E3 ubiquitin ligase MDM2. E3 ubiquitin ligases exert their activities through the covalent linkage of a ubiquitin molecule to a substrate which, depending upon the number of ubiquitins and the specific lysine to which subsequent ubiquitins are linked, signals one of many outcomes for the substrate. The transfer of a ubiquitin molecule to a substrate involves the concerted activity of at least three enzymes: the ubiquitin-activating enzyme E1, which is conjugated to ubiquitin via a high-energy thioester bond; a ubiquitin-conjugating, or E2, enzyme, to which the E1-bound ubiquitin is transferred; and an E3

which, depending on its class, directly or indirectly catalyzes the transfer of the E2-bound ubiquitin to a substrate lysine (Pickart, 2001). Generally, ubiquitin molecules linked by K48 to G76 of a subsequent ubiquitin molecule tend to direct the substrate for proteasomal degradation when a sufficient chain length, usually four, is reached. In contrast, K63 to G76 linked ubiquitin chains signal various non-proteasomal fates for their substrates (Pickart et al., 1999; Spence et al., 2000).

In the case of p53, MDM2 is a RING finger E3 that is well known to direct the addition of K48-linked ubiquitin to several C-terminal lysines of p53 and to occlude the p53 transactivation domain (Haupt et al., 1997; Honda et al., 1997; Momand et al., 1992). In conjunction with E4 (ubiquitin-chain extending) 0.ubiquitin ligase activities of the p300 and CBP acetyltransferases, K48 ubiquitin chains are extended on p53, targeting it for degradation by the proteasome (Grossman et al., 2003a; Shi et al., 2009b). Validating the importance of MDM2 in keeping p53 in check was the observation that embryonic lethality in MDM2-null mice can be completely rescued by concomitant deletion of p53 (Jones et al., 1995; Luna et al., 1995), implying that a primary function of MDM2 is to control p53 activity under non-stress conditions. Furthermore, MDM2 is overexpressed in many sarcomas which, in many cases, is mutually exclusive with p53 mutation (Florenes et al., 1994), suggesting that MDM2 overexpression can often render p53 signaling inactive (although many p53-independent activities of MDM2 likely contribute to tumorigenesis as well).

MDM2 antagonizes p53 activity primarily by promoting its ubiquitination and, independently, attenuating p53 transcriptional activity through direct interaction with

p53. Both crystalization and biochemical structure/function analysis of the p53-MDM2 complex have yielded insights into the nature of this interaction, which involves an N-terminal hydrophobic cleft of MDM2 spanning aa 25-109 interacting with a small hydrophobic portion of the p53 transactivation domain spanning aa 19-26 (Chen et al., 1993; Kussie et al., 1996). Additionally, the p53 C-terminal tetramerization domain is also necessary for efficient MDM2 binding (Maki, 1999). This interaction with the p53 N-terminus serves to occlude the p53 transactivation domain, thereby rendering it transcriptionally inactive while in complex with MDM2 (Kussie et al., 1996). Phosphorylation events in the p53 N-terminus, which will be discussed in more detail below, serve to weaken the p53-MDM2 interaction through reduction of hydrophobicity of N-terminal residues (Schon et al., 2002), thereby antagonizing the negative regulatory functions of MDM2 toward p53. While the outcome of p53-directed ubiquitination by MDM2 is highly context dependent, it is likely that ubiquitination negatively regulates p53 activity through affecting p53 localization (Stommel et al., 1999), transcriptional activity (Brooks and Gu, 2006), competing against other post-translational modifications, or directly promoting degradation by facilitating the delivery of p53 to the 26S proteasome (Brignone et al., 2004; Kulikov et al., 2010).

Further work has suggested a complex stoichiometry of the interaction – MDM2 oligomerization appears necessary to activate its E3 activity, and this oligomerization event is likely dependent on additional factors including the close MDM2 homolog MDM4 (Linke et al., 2008). The spatial organization of the MDM2 oligomer is a tail-to-RING domain interaction between neighboring MDM2 molecules, which facilitates the

activation of the E3 activity of the RING domain and substrate ubiquitination. This change in activity may be facilitated through augmentation of MDM2-E2 binding (Poyurovsky et al., 2007).

In addition to the complex biochemical interplay between p53 and MDM2, there exists another level of genetic complexity to the interaction: MDM2, the primary negative regulator of p53 activity, is itself a high-affinity p53 target gene (Barak and Oren, 1992; Otto and Deppert, 1993; Wu et al., 1993). This interaction generates a negative feedback loop whereby stress-induced activation of p53 culminates in induction of MDM2, which then serves to dampen p53 activity. It is the interruption of this feedback loop by the inability of mutant p53 to transactivate MDM2 that results in its overexpression in many cancers. The physiological significance of this feedback loop is evidenced by the fact that in many cancers retaining wild-type p53, MDM2 is overexpressed, suggesting that either p53 mutation or MDM2 overexpression achieve an outcome of hampering p53 activation (Daujat et al., 2001). Precisely how the MDM2-p53 interaction is regulated by upstream p53-activating signals will be described in more detail below.

Upstream Activators of the p53 Program

p53 is a potent sensor of an ever-increasing range of cellular stresses; DNA lesions from a broad spectrum of environmental or chemical sources, hyperproliferation, improper timing of activation of many protooncogenes, hypoxic conditions, and telomere erosion are just a few of the stresses that lead to p53 stabilization and activation. In the

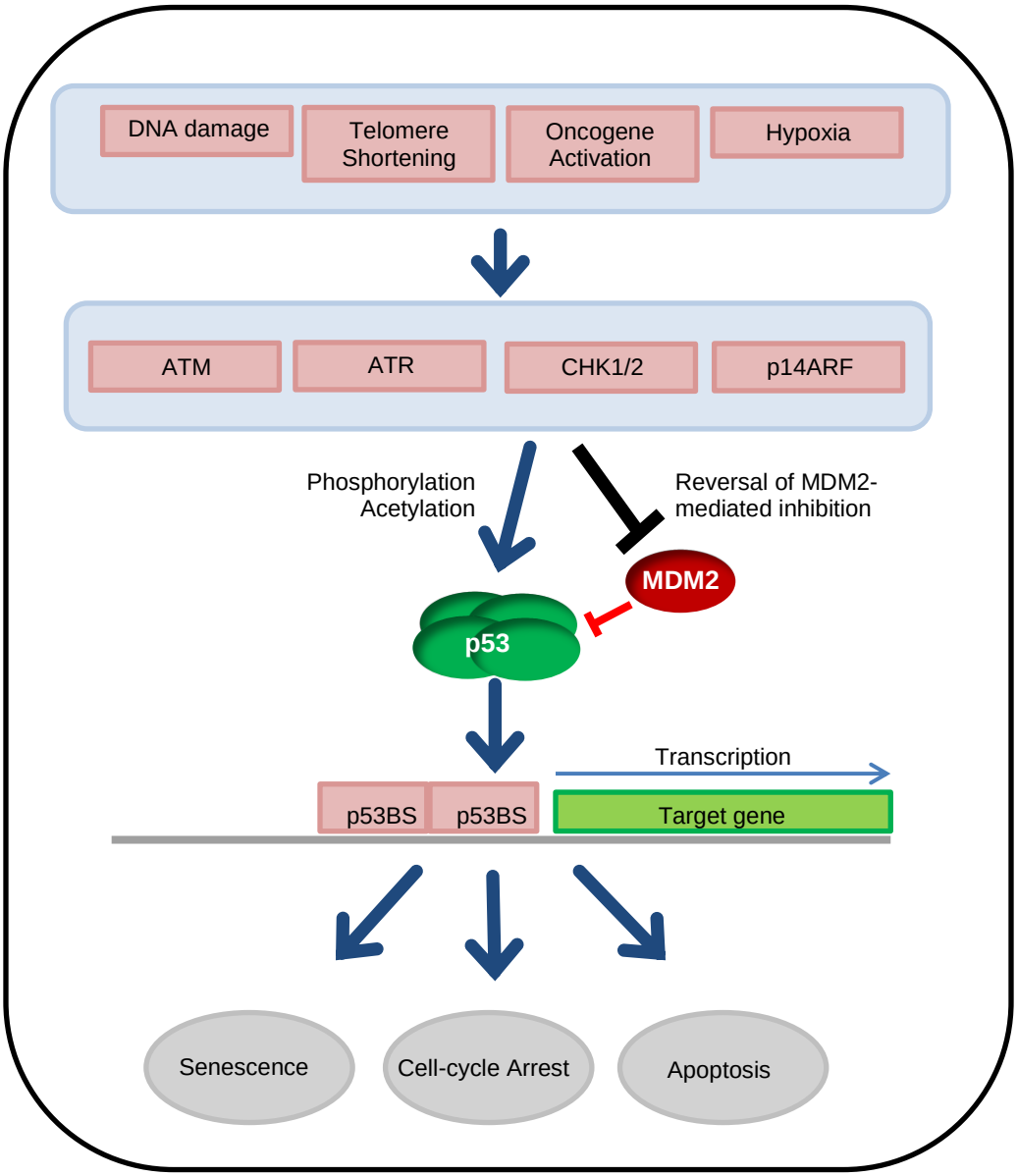
past few years alone, p53 has been linked to the tanning response (Cui et al., 2007), defined as an antagonist of induced pluripotent stem-cell (iPS cell) generation (Banito et al., 2009; Marion et al., 2009), and even as a regulator of embryonic implantation (Hu et al., 2007). Still, the most well-studied and, arguably, relevant functions to tumor initation and progression, are the p53-dependent responses to DNA-damage and oncogene activation (Figure 1.1).

DNA-damage

Numerous chemical and environmental, and even normal metabolic causes of DNA damage exist in nature. Oxidative respiration causes the generation of reactive oxygen species (ROS), which cause base modifications in DNA that have various, usually detrimental effects. Ultraviolet radiation also contributes to the development of some tumors (Brash et al., 1991) through the generation of pyrimidine dimers which commonly result in base substitution. These lesions can result in point mutations and chromosomal aberrations that can often result in oncogene expression or tumor suppressor inactivation, underscoring the need for a rapid, robust response to these threats to genomic integrity. The potent G1 arrest mediated by p53 in response to various types of DNA damage is mediated primarily by the ATM and ATR kinases, with Akt and the DNA-dependent protein kinase (DNA-PK) being additionally necessary for p53 accumulation in response to sources of high frequency gamma irradiation (Boehme et al., 2008).

Figure 1.1: The p53 Tumor Suppression Pathway. An overview of the p53 pathway including p53-activating stresses such as DNA damage, oncogene activation, telomere attrition, or hypoxia (top row), the transducers of these stress signals to p53 (second row), and the physiologic outcomes directed by activated p53 (grey ovals), which can include a transient or permanent cell-cycle arrest, or apoptosis.

Figure 1.1: The p53 Tumor Suppression Pathway



Adapted from Riley *et al* 2008, Nature Reviews Molecular and Cell Biology

ATM/ATR and DNA-PK sit at the top of the double-strand break response pathway. ATM is recruited as a dimer to double-strand breaks (DSBs) by the Mre11/Rad50/Nbs1 (MRN) complex. Once recruited to DSBs, ATM undergoes an autophosphorylation which results in the dissociation of the ATM dimer, yielding ATM monomers with kinase activity (Bakkenist and Kastan, 2003) which serve to phosphorylate a host of substrates in the DNA-damage response. ATR seems to function analogously to ATM, but responds to a different subset of DNA lesions such as single-strand breaks generated by UVB radiation (Zou and Elledge, 2003) and, in contrast to ATM, appears to be necessary for normal cell-cycle progression (Brown and Baltimore, 2003). DNA-PK is activated directly through its autophosphorylation in the presence of DSBs (Smith and Jackson, 1999).

In response to the activation of any combination of these kinases, p53 is phosphorylated at several serine residues, most notably serine 15. In addition, DNA-PK also phosphorylates p53 at serine 37 and 46 (Hill et al., 2008; Komiyama et al., 2004; Leesmiller et al., 1992), while ATM and ATR also target serine 20 for phosphorylation indirectly through the activation of the checkpoint kinases Chk1 and Chk2 (Donehower et al., 1992). Phosphorylation of these residues, located in the N-terminal transactivation domain of p53, may serve to disrupt the p53-MDM2 interaction, although more work is necessary to rigorously test this hypothesis. In addition, ATM phosphorylates the MDM2 C-terminus, stimulating its autoubiquitination, thereby targeting it for proteasomal destruction (Stommel and Wahl, 2005) and allowing stabilization of p53 through a reduced rate of proteasome-dependent turnover.

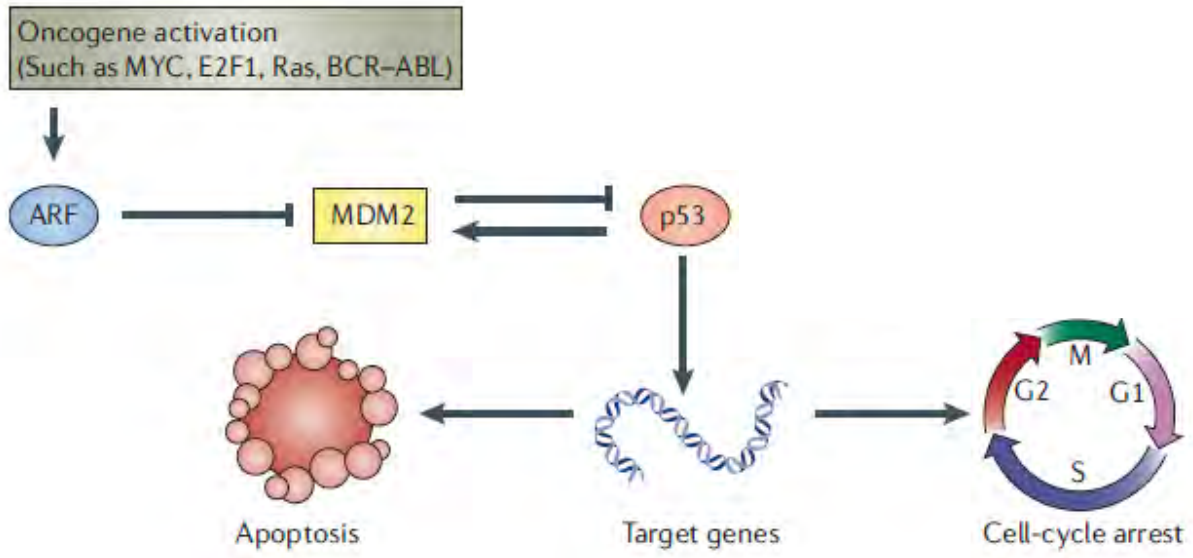
Oncogenic Stress: p14ARF

The threat of extensive DNA-damage to the health of an organism is unquestionable; however, to our understanding the broadest purpose of tumor suppression is to prevent the improper activation of proto-oncogenes which could constitute a threat to the organism as a whole. While DNA-damage alone can, and often does, result in the activation of oncogenes through amplification or mutational events, other DNA-damage independent paths exist to oncogene activation. This necessitates the need for a robust response to these events, even in the absence of genetic lesions. In vertebrates, this response is largely controlled by the gene products of the INK4A locus.

The INK4A locus, located at chromosome 9p21 in humans, encodes two potent tumor suppressors, p16INK4A and ARF, encoded in alternative reading frames, which are therefore structurally unrelated. ARF, aptly named the alternative reading frame of the locus, is the product of a promoter and first exon (exon 1β) that is unique from p16INK4A, but is then spliced into an alternate reading frame of common exons 2 and 3 of p16INK4A (Quelle et al., 1995). Interestingly, although the locus is conserved throughout vertebrate evolution, only the first 25 amino acids of ARF, corresponding to exon 1β, are conserved. This suggests that evolutionary relevant functions of ARF are provided by the first 25 amino acids (Kim et al., 2003), although the identification of a nucleolar localization signal (NoLS) in exon 2 which regulates SUMOylation of MDM2 may challenge this assumption (Xirodimas et al., 2002).

Figure 1.2: Overview of the ARF Signaling pathway. ARF is expressed in response to several oncogenic stimuli, and activates p53 by directing MDM2 degradation and nucleolar relocalization. Depending upon environmental context, p53 directs a pro-apoptotic or cell-cycle arrest program in response to ARF

Figure 1.2: Overview of the ARF Signaling pathway



Adapted from Sherr, CJ 2006, *Nature Reviews Cancer*

p16INK4A had been previously characterized as an antagonist of CDK4/6 activity through inhibition of cyclin-D binding, leading to Retinoblastoma (Rb) hypophosphorylation and inhibition of cell-cycle progression (Serrano et al., 1993). Furthermore, before the discovery of ARF, deletion of the locus had already been observed at a high rate in a variety of malignancies, suggesting a tumor suppressive activity at 9p21 (Sharpless and DePinho, 1999). ARF was soon characterized and also discovered to harbor a potent cell-cycle inhibitory activity (Quelle et al., 1995) at both G1/S and G2/M phases, as evidenced by the upregulation of p21 and 14-3-3σ in response to forced ARF expression (Hemmati et al., 2008). These properties appeared to confer tumor suppressive capacity, as mice engineered to lack exon 1β (but retain p16INK4A expression) were shown to be highly tumor prone, suggesting that mouse ARF, not p16INK4A, may be the relevant tumor suppressor at 9p21 (Kamijo et al., 1997).

While mice lacking ARF are given to spontaneous tumor formation, evidence for the role of human ARF versus p16INK4A in tumor suppression is less well-defined. Complicating these studies in part is the architecture of the locus itself, the entirety of which is often lost in most cancers. When mutations in the INK4A locus do occur in human cancer, they often occur with concomitant loss of function of both ARF and p16INK4A, making assessment of the relative contribution of each to the malignancy difficult. However, most hypermethylation or deletion events that are selective for one of the two gene products target p16INK4A in humans, whereas selective ARF inactivation is much (about a factor of 20) more rare (Kim and Sharpless, 2006; Sharpless and Chin, 2003). Still, a few lines of research suggest a role for selective loss of ARF in human

tumor development. First, a group of familial melanoma and astrocytoma kindreds exhibit exon 1β mutations that, as expected, do not affect p16INK4A function (Rizos et al., 2001). Additionally, inactivation of ARF by promoter hypermethylation in the absence of p16INK4A hypermethylation has been shown to occur in colon cancers (Esteller et al., 2000; Sato et al., 2002) and Merkel cell carcinoma (Lassacher et al., 2008). Additionally, immortalization of cultured mammary epithelial cells (MECs) by high-risk HPV E6 is closely associated with loss of ARF signaling.

Differences in activation of the gene products between mice and humans are more pronounced. As each factor encoded by INK4A is a potent cell-cycle inhibitor, activation of each must be tightly regulated to allow cell-cycle progression under normal conditions. This is achieved primarily through the regulation of expression of each or both factor(s) by upstream oncogenic signals (although, importantly, both products have been noted as targets of MDM2-independent polyubiquitination and proteasomal degradation, suggesting possible post-transcriptional regulation of each) (Ben-Saadon et al., 2004; Kuo et al., 2004). In mice, expression is largely coregulated (Krishnamurthty et al., 2004), with both senescence or activated Ras resulting in increases of both ARF and p16INK4A expression levels. In humans, however, p16INK4A alone appears to respond to replicative senescence and activated Ras (Huot et al., 2002).

Further analysis of p14ARF, however, has proven that its cell-cycle regulatory properties are more complex. While normal replicative senescence does not appear to activate ARF in humans, oncogene-induced senescence appears to be regulated by ARF in certain contexts (Sekaric et al., 2007a). Additionally, E2F1 and Ras were observed to

synergistically activate p14ARF in primary fibroblasts (Berkovich and Ginsberg, 2003). One surprising study failed to detect p14ARF in passaged primary fibroblasts, yet siRNA-mediated knockdown of p14ARF increased the growth rate of these cells in a p53-dependent manner. Knockdown of p16INK4A in the same experimental setting provided a growth advantage only with concomitant loss of p14ARF, suggesting that combined loss of the entire locus contributes to an enhanced growth rate of human fibroblasts (Voorhoeve and Agami, 2003). While marked differences exist between ARF regulation in mice and humans, much more comprehensive analyses are required to discern precisely how, and under what conditions, p14ARF is activated.

Once expressed through the action of the oncogenic signaling pathways described above, p14ARF exerts a subset of its activities through p53 stabilization and p21 induction in a complex and still incompletely understood manner (Figure 1.2). Notably, p14ARF has been shown to possess p53-independent cell-cycle inhibitory and apoptotic functions in part through the CtBP family of transcriptional repressors (Kovi et al., 2010; Paliwal et al., 2006), but these activities will not be discussed in detail. The process likely begins with p14ARF forming a ternary complex with MDM2 and p53, inhibiting the E3 ligase activity of MDM2 toward p53 (Xirodimas et al., 2001), and effecting the rapid proteasomal degradation of MDM2. This p53-ARF interaction requires the presence of MDM2 and the N-terminal exon 1β-encoded amino acids of ARF, suggesting that the ARF N-terminus interacts with MDM2, which bridges the ARF-p53 interaction (Zhang et al., 1998). ARF interaction with MDM2 appears necessary for the stabilization of p53, and appears to occur through multiple domains of MDM2 (Clark et al., 2002).

An additional complexity of the MDM2-ARF axis arises from the many reports that ARF is predominantly nucleolar. Furthermore, in the presence of ARF, MDM2 is also localized to the nucleus, suggesting that ARF may serve to either inhibit nucleolar-cytoplasmic shuttling of the MDM2-p53 complex (Tao and Levine, 1999), or to directly sequester ARF-MDM2 complexes in the nucleolus (Weber et al., 1999). The authors noted that ARF mutants failing to reach the nucleolus but still capable of interacting with MDM2 were unable to activate p53, supporting the notion that nucleolar localization of ARF is necessary for MDM2 inhibition; however, several challenges to this hypothesis indicate a more complex picture. Work from the lab of Gordon Peters noted several cases in which non-nucleolar ARF can direct p53 stabilization despite relatively high levels of nucleoplasmic MDM2 (Llanos et al., 2001). Subsequent work from the same lab indicated that nucleolar ARF is highly stable relative to nucleoplasmic ARF, raising the possibility that nucleolar ARF serves as a potential reservoir for nucleoplasmic ARF, where it may predominantly exert its critical functions (Rodway et al., 2004).

Once released from MDM2-mediated ubiquitination and transcriptional inhibition by p14ARF, p53 is stabilized and, through mechanisms still under investigation, becomes active. p14ARF was recently shown to direct the acetylation of p53 in both the DNA-binding domain at lysine 120 (Mellert et al., 2007), and the C-terminus (Sekaric et al., 2007a). Lysine 120 is a known substrate of Tip60/hMOF-mediated acetylation, but the physiologic HAT responsible for ARF-induced lysine 120 acetylation has not yet been definitely identified. While C-terminal acetylation of p53 is known to involve the p300 and CBP acetyltransferases (Luo et al., 2004), work from the Androphy lab has presented

convincing evidence that the transcriptional adaptor hAda3, a stable component of the P/CAF complex (Ogryzko et al., 1998), regulates ARF-induced acetylation of p53 C-terminal lysines. Strikingly, siRNA-mediated ablation of hAda3 completely abrogated p53 stabilization in response to ectopic p14ARF, suggesting that acetylation may be necessary for p53 stabilization (Sekaric et al., 2007a). This dissertation will present evidence that P/CAF may indeed be the HAT responsible for acetylation of the p53 C-terminus, and this function is critical in promoting p53 stabilization in response to p14ARF.

In addition to the complex interplay between p14ARF and MDM2 in p53 activation, one report suggests that p14ARF must also inactivate another E3 ubiquitin ligase – ARF-BP1/Mule – for p53 stabilization. This report indicates that this ubiquitin ligase, identified as an E3 ligase for the anti-apoptotic factor Mcl-1 (Zhong et al., 2005), also harbors E3 activity toward p53, the ARF-dependent inhibition of which is critical for proper p53 activation (Chen et al., 2005). The potential therapeutic importance of ARF-BP1/Mule is also underscored by the observation that its targeted inhibition in a variety of cancer cells induces cell-cycle arrest or apoptosis (Chen et al., 2006). Further work will be necessary to more accurately define the complex biochemical network that coordinates the tumor suppressive activities of ARF.

Coactivators of p53-dependent Transcription

In the context of the complex chromatin environment that houses the genome, eukaryotes have evolved mechanisms to tightly regulate the temporal activation of

transcription at many loci. Stress-induced factors which present an existential threat to the cell, such as many transcriptional targets of p53, make this need abundantly clear. In eukaryotes, one level of transcriptional control is provided a large, structurally and functionally heterogeneous class of proteins known as transcriptional coregulators. While these factors lack DNA-binding domains, they are capable of interacting with transcription factors to enhance or repress expression of their target genes.

Transcriptional coactivators, which are well studied with respect to p53, broadly function to disrupt higher-order chromatin structure and nucleosome-DNA interactions to allow transcriptional initiation and elongation. These factors fall into one of two major groups: histone acetyltransferases (HATs) serve to facilitate transcriptional activation through acetylation of histone or non-histone factors, whereas ATP-dependent chromatin remodeling proteins of the mammalian homologs of the yeast SWI/SNF complex disrupt nucleosome-DNA interactions. While the SWI/SNF proteins are critical for the coordination of regulated transcription and should be studied in more detail with regard to p53-dependent transcription, the bulk of research on p53 coactivators has focused on the role of HATs in regulation of p53 activity.

The Many HATs of p53

Since four simultaneous communications highlighted the role of the paralogous HATs p300 and CBP as p53 coactivators (Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997; Scolnick et al., 1997), numerous HATs with various activities toward p53 have been described. Far from playing a simple coactivator role in the activation of p53 target

genes by acetylating histones, several p53 HATs are now known to alter p53 activities through direct acetylation (Gu and Roeder, 1997; Tang et al., 2006), through novel E3 or E4 (ubiquitin-chain extending) ligase activities (Grossman et al., 2003a; Linares et al., 2007; Shi et al., 2009a), or by direction of these biochemical activities toward other modulators of the p53 pathway (Linares et al., 2007; Shin and Janknecht, 2007; Wang et al., 2004). While the bulk of data in this dissertation concerns the impact of P/CAF on p53 stability and p21 transcription, p300/CBP and Tip60/hMOF play important roles in these processes (Figure 1.3) which need to be considered in the development of a comprehensive model for the role of HATs in p53 function.

p300 and CBP

p300 and CBP are unquestionably the most well-studied HATs with respect to p53 function. As mentioned above, these factors were defined as p53 coactivators, likely owing both to their ability to bridge interactions between transcription factors and RNA Polymerase II (RNAPII) (Goodman and Smolik, 2000), and through their acetylation of promoter histone tails. Initial characterization of p300 and CBP histone acetylation activity identified four lysines of the N-terminal histone 4 (H4) tail as substrates (Ogryzko et al., 1996). Recent *in vivo* analysis of p300/CBP double-knockout MEFs confirms this observation, as H4 acetylation at all four N-terminal lysines – K5, K8, K12, and K16 – was strongly attenuated at known p300/CBP-responsive genes (Kasper et al., 2010).

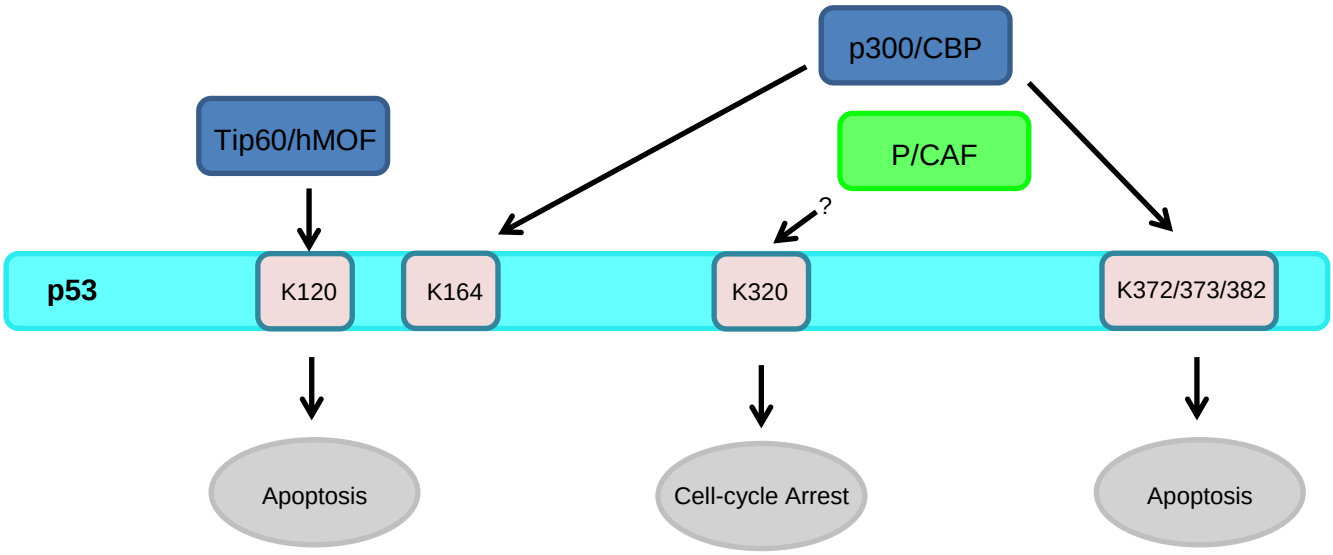
This broad role for p300/CBP in transcriptional activation extends to p53, as evidenced by the requirement for p300 in directing the p53-dependent *in-vitro* transcriptional activation of a purified, chromatin-assembled p21 promoter (Espinosa and Emerson, 2001). Importantly, the relevant function of p300 in this study was acetylation of nucleosomal histones; p53 acetylation was dispensable for p21 activation, at least in this context, although it is possible that other non-structurally related HATs such as Tip60/hMOF or P/CAF/GCN5 could obviate the requirement for p300 in this experimental setting. Importantly, CBP recruitment to the p21 promoter following irradiation of U2OS cells
correlates with increased histone acetylation (Barlev et al., 2001).

Complicating the simple hypothesis that p300 and CBP function as histone acetyltransferases for p53 are several reports indicating that p300 or CBP are either completely dispensable for p21 transactivation (Kasper et al., 2010), or that their HAT activities are not necessary for p21 transactivation (Puri et al., 1997). The latter report suggests that P/CAF provides the necessary HAT activity, functioning through interaction with p300/CBP to facilitate activation of p21. The role of CBP and p300 in p53-dependent transactivation of the p21 promoter is likely extremely complex and context specific, and will require a great deal more research to fully understand. The involvement of P/CAF in p21 expression will be described further below.

A less controversial function of p300 and CBP activity in p53 function is the direct acetylation of p53 by p300 and CBP. p53 was identified by mass spectrometry as

Figure 1.3: Impact of p53 acetylation by Tip60/hMOF, p300/CBP, or P/CAF on cell fate. Acetylation of K120 by Tip60/hMOF results in increased association of p53 with low-affinity apoptotic promoters. K320 acetylation, possibly mediated by P/CAF, is associated with cell-cycle arrest. Acetylation of C-terminal lysines by p300 and CBP increase association with apoptotic promoters. The role of the recently described acetylation site at K120 remains unclear but, when mutated in combination with the other seven acetylation sites, abolishes p21 activation.

Figure 1.3: Impact of p53 acetylation by Tip60/hMOF, p300/CBP, or P/CAF on cell fate.



the first non-histone protein to be acetylated by a HAT. p53 is acetylated by p300/CBP at six C-terminal lysines surrounding the tetramerization domain(Gu and Roeder, 1997), as well as in the DNA-binding domain at K164(Tang et al., 2008). Subsequent studies suggested numerous functions for acetylation of the p53 - augmentation its DNA-binding capability (Luo et al., 2004), inhibition of ubiquitination of C-terminal lysines(Li et al., 2002), facilitation of cofactor recruitment (Barlev et al., 2001). Mutation of the six C-terminal lysines (6KR) acetylated by p300 and CBP result in attenuated expression of a subset of p53 targets (Feng et al., 2005), suggesting that acetylation of these residues play an important role in p53-dependent transcription. Strikingly, mutation of K164 and the Tip60 target K120 in combination with 6KR completely impair p53-regulated cell-cycle arrest and apoptosis, with only the p53-MDM2 feedback loop remaining intact (Tang et al., 2008). These observations further underscore the importance of p53 acetylation in its activation, and suggest that p21 expression is, in a manner yet to be completely elucidated, dependent upon some degree of p53 acetylation.

p300 and CBP have been historically known as coactivators through their acetylase activities, but p300/CBP have also been shown to play in important role in promoting p53 degradation (Grossman et al., 2003a) (Shi et al., 2009a). Early lines of evidence indicated that p300 is present in MDM2-p53 complexes, and that an MDM2 mutant deficient in p300 binding is unable to degrade p53, despite retaining robust p53 interaction, proper localization, and containing an intact RING domain (Grossman et al., 1998). Furthermore, expression of adenovirus E1A has long been known to stabilize p53 in a manner dependent upon p300/CBP binding (Somasundaram et al., 1997). The

definitive role for p300 in p53 stability was described when p300 was shown to exhibit intrinsic autoubiquitination activity and ubiquitin ligase activity toward p53 *in vitro*. Importantly, p300 was only able to ubiquitinate monoubiquitinated p53, designating it as an E4 ubiquitin ligase (Grossman et al., 2003a). Subsequent work also identified CBP as an E4, and validated the roles of each in p53 degradation *in vivo.* Interestingly, the authors noted that purified cytoplasmic p300 and CBP exhibited robust E4 ubiquitin ligase activity, while nuclear p300 and CBP lacked E4 activity. This observation could potentially reconcile the opposing functions of p300 and CBP in regulating p53 activity.

Continuing research will be necessary to determine whether the negative regulatory activities of p300/CBP toward p53 are limited to non-stress conditions, or if p300/CBP-directed ubiquitination of p53 serves additional purposes; perhaps in promoter clearance, restoration of low p53 levels following stress, transcriptional regulation, or any number of other possibilities. Additionally, the positive-acting roles of p300 and CBP as coactivators of p53-directed transcription will require further attention – RNAi approaches should allow the dissection of which subset(s) of p53-responsive genes require the presence of p300 or CBP as a coactivator.

Tip60 and hMOF

Tip60 and hMOF are members of the evolutionarily conserved family of 'Moz,Ybf2/Sas3,Sas2, and Tip60' (MYST) histone acetyltransferases. Tip60 is involved in numerous cellular processes such as DNA damage, apoptosis (Ikura et al., 2000), and maintenance of stem cell identity (Fazzio et al., 2008). Tip60 exerts most of these

activities in part through acetylation of H4 at lysines 5, 8, 12, and 16, as well as a number of non-histone targets(Sapountzi and Cote, 2011). Little is known about hMOF, but it too appears to play a role in the DNA-damage response through association with ATM and acetylation of H4 at lysine 16 (Gupta et al., 2005; Taipale et al., 2005). Recently, Tip60 and hMOF were both independently identified to modulate p53 activity through acetylation of K120 (Sykes et al., 2006; Tang et al., 2006), a highly conserved residue present in the DNA-binding domain. Strikingly, both reports noted that acetylation of K120 seemed to regulate apoptosis induction by p53, as its mutation attenuated p53 recruitment and transcription at apoptotic promoters such as PUMA and Bax, but had no effect on induction of p21 and MDM2. Of note, Tip60 appeared to play a critical role in acetylation of H4 at the p21 promoter – while the p53 K120R mutant retained the ability to transactivate the p21 promoter, Tip60 knockdown strongly attenuated p21 expression in response to etoposide. Further analysis indicated that Tip60 was recruited to the p21 promoter following stress, and its presence correlated with acetylation of H4.

P/CAF and GCN5

P/CAF and GCN5 are members of the GCN5-related N-acetyltransferase, or GNAT, family of histone acetyltransferases. Both mammalian P/CAF and GCN5 C-terminal halves bear strong sequence similarity to yeast GCN5 and contain an evolutionarily conserved HAT domain. Metazoan P/CAF and GCN5, however, have evolved a long N-terminal extension whose function was elusive until the recent report

that it encodes an intrinsic E3 ubiquitin ligase activity, which will be described in more detail below. P/CAF and GCN5 function in large multi-protein complexes – the 2MDa STAGA/SAGA and P/CAF complexes, which seem to share most subunits but differ in whether their catalytic activity is provided by P/CAF or GCN5; and the smaller ~700kD ATAC complex, which may share similarity to the yeast ADA complex (Nagy and Tora, 2007).

The GCN5 and P/CAF complexes have a relatively broad substrate specificity for lysines of histone 3. *In vitro* and *in vivo* studies indicating that H3K14 is a primary substrate of GCN5 (Brand et al., 1999; Guelman et al., 2006) are supported by X-ray crystallographic data indicating that an 11-amino acid peptide of H3 centered around K14 fits closely into a protein cleft formed by the folded structure of the GCN5 core domain in combination with both the N- and C-termini (Rojas et al., 1999). More limited research has focused on P/CAF, but broad agreement exists that N-terminal tails of histone 3 are primary substrates of the P/CAF complex *in vitro* and *in vivo* (Ogryzko et al., 1998).

The first evidence that P/CAF plays a role in p53 transactivation arose from the observation that transfected P/CAF enhanced transactivation of the p21 promoter(Scolnick et al., 1997), presumed to occur through enhancement of nucleosomal histone acetylation. Two subsequent studies dissecting the complex series of signals that culminate in p53 activation suggested that, in addition to the acetylation of C-terminal lysines, lysine 320 is acetylated following UV or ionizing radiation. It was noted that P/CAF efficiently acetylated p53 lacking the C-terminal regulatory domain, while p300 was unable to acetylate this mutant (Liu et al., 1999). Importantly, mass spectrometric

analysis of *in vitro*-acetylated p53 also confirmed this result(Sakaguchi et al., 1998), as did later reports indicating that overexpression of P/CAF stimulates acetylation of p53 K320 in cells (Jin et al., 2002; Linares et al., 2007). To date, and perhaps surprisingly, there have been no reports of p53 acetylation by GCN5.

Several lines of evidence to date suggest that P/CAF and, to a lesser extent, GCN5 play an important role in p53-directed stress responses. In addition to the first report that P/CAF stimulates p21 expression, mentioned above, two recent reports indicate that P/CAF is necessary for full expression of p21 (Di Stefano et al., 2005b; Linares et al., 2007). In addition, despite the paucity of literature addressing GCN5 in p53 activity, one report from the Roeder lab suggests that the GCN5-containing STAGA complex enhances p53-dependent transcription, as GCN5 knockdown partially inhibits UV-induced activation of PUMA and p21. Furthermore, GCN5 interacts with p53 in cells and is recruited to active promoters with p53, correlating with acetylation of histone 3(Gamper and Roeder, 2008). These lines of evidence suggest that P/CAF and GCN5 function as coactivators in p53-dependent transcription of a subset of target genes, albeit in an incompletely understood manner.

Acetylation of p53 K320 has been presumed to be the primary manner through which P/CAF regulates p53. This modification regulates diverse cellular processes – mutation of K319-321 to arginines results in attenuated transcriptional activation of a p21-luciferase reporter (Liu et al., 1999). In addition, expression of a K320Q acetylation mimic in p53-null H1299 cells exhibits markedly different effects on p53-responsive promoters than expression of a wild-type or K373Q mutant. One comprehensive report

indicates that K320Q is not able to induce expression of most apoptotic genes, yet strongly activates cell-cycle arrest and anti-apoptotic promoters; in contrast, K373Q strongly activates many apoptotic genes such as APAF1, caspase-6, and PIG-3 while actively repressing expression of many pro-survival genes (Knights et al., 2006). Further analysis indicates that K373Q was also efficiently recruited to apoptotic promoters, whereas K320Q bound strongly to the p21 promoter but not apoptotic, low-affinity promoters. These results suggest that acetylation of K320 may prevent activation of several low affinity p53-responsive promoters, thereby modulating the decision between cell-cycle arrest and apoptosis.

Despite mounting evidence suggesting that K320 acetylation is a biologically relevant modification of p53, and that P/CAF overexpression stimulates this modification *in vitro* and in cells, the true mediator of K320 acetylation remains unclear; despite numerous reports of P/CAF overexpression stimulating this modification, no reports to date have addressed the impact of HAT depletion on K320 acetylation, leaving open the possibility that P/CAF is a limiting adaptor and may function through other mechanisms to modulate p53-dependent transcription. Many of the experiments described in this dissertation will address the mechanism through which P/CAF promotes p53-dependent p21 transcription in response to various stresses.

Transcriptional Regulation by p53

p53 was formally identified as a transcriptional activator with sequence specificity in 1992 by Bert Vogelstein and colleagues. The group identified the p53 consensus

binding sequence as two inverted RRRCWWGYYY repeats separated by a short (0-21bp) spacer(el-Deiry et al., 1992). Since this definition, many sequence-based biochemical and computational approaches have been undertaken to predict and functionally validate potential p53 target genes; a 2006 report utilizing a global chromatin-immunoprecipitation strategy identified 542 p53-binding sites, while more recent approaches have even implicated p53 in the direct activation of microRNAs, further expanding the list of p53 transcriptional targets.

p53 directs an appropriate cell-fate outcome in part through differential activation of target genes in a cell-type and stress-specific manner (Espinosa, 2008), implying that the p53 transcriptional program is tightly regulated. In general, p53 is described as directing an outcome of transient cell-cycle arrest or apoptosis, with variations such as senescence, a permanent G0 arrest, often considered separate outcomes. Early experiments dissecting the transcriptional p53 response indicated that kinetics of induction of p53 differed, with cell-cycle arrest genes broadly displaying fast, often transient expression; while apoptotic genes generally display delayed but sustained expression kinetics (Zhao et al., 2000). Over the years, a variety of hypotheses have been proposed to explain the specificity of p53 in target gene regulation.

It was initially believed that the activation status of target promoters correlated with the presence of p53 at the p53 REs of those promoters. This hypothesis met with some support owing to differential binding affinity of p53 REs in different subsets of p53-regulated genes; however, mounting evidence suggests that promoter occupancy by p53 of p21, a target gene responsible for inducing cell-cycle arrest, reflects the total

cellular concentration of p53 irrespective of the transcriptional state of the p21 promoter (Espinosa et al., 2003). Specifically, stresses that resulted in p53 stabilization also resulted in similar levels of p53 recruitment to the p21 promoter, while only a subset of these stresses resulted in sustained transcription of p21. Further challenging this hypothesis was the observation that p53 occupancy of target promoters measured in a p53 ChIP microarray was static in response to different stresses, yet expression of these factors varied in a stress-specific manner (Wei et al., 2006). While the hypothesis may hold true when applied to individual target genes in specific contexts, it is an insufficient explanation for the specificity of p53 transcriptional regulation as a whole.

Another early hypothesis arose out of the observation that different stress stimuli direct different arrays of post-translational modifications on p53, presenting the possibility that p53 transcriptional programs are a product of the relative prevalence of differentially modified p53 isoforms. A wealth of work on specific modifications of p53 and their cell-fate preferences provides some support for this model. Tip60 and MYST acetyltransferases, for example, promote acetylation of lysine 120 of p53, an event that is critical for induction of p53-mediated apoptosis, but dispensable for growth arrest (Tang et al., 2006). Similarly, phosphorylation of serine 46 appears necessary for activation of the apoptotic gene *p53AIP1,* yet again dispensable for p21 activation (Oda et al., 2000). Many similar examples exist of modifications provided by specific factors which are necessary for activation of pro-apoptotic promoters, yet dispensable for cell-cycle arrest. These observations collectively raise the hypothesis that many modifications of p53 serve to provide a more strongly activating environment in the vicinity of low-affinity

promoters - either through increasing DNA-binding capabilities, p53 levels, or interactions with coactivators or other modulators.

Again, however, recent evidence challenges the idea that the p53 'bar code' of post-translational modifications provides a universal explanation for promoter selectivity in transcriptional activation. A pharmacological inhibitor of the p53-MDM2 interaction, nutlin-3 (Vassilev et al., 2004), has been widely used over the past few years to study the consequence of p53 stabilization in the absence of canonical stress stimuli. Using nutlin-3, cell-fate decisions effected by p53 believed to require specific arrays of p53 modifications were shown to occur even in the absence of these modifications (most significantly, serine-15)(Thompson et al., 2004). While the code may, and most likely does, contribute to p53-dependent transcriptional cell-fate decisions, it again is insufficient to fully account for the complexity of the p53 transcriptional response.

Several recent analyses have suggested that the context of the promoter-associated transcriptional complexes control the regulation of certain p53 target genes at steps downstream of p53 and RNAPII binding(Gomes and Espinosa, 2010a). The most comprehensive analysis of transcriptional machinery binding at the p21 promoter (Donner et al., 2007) capitalizes on the observation that two robust stabilizers of p53 in HCT116 cells, nutlin-3 and UV, differentially activate p21 expression – nutlin-3 induces sustained transcription of p21, while UV produces a weak, transient elevation of p21 levels. Observation by ChIP analysis indicated that p53 and RNAPII occupy the p21 promoter to similar degrees in both scenarios, failing to explain the difference in p21 expression status. Further analysis of transcriptional machinery present at the promoter

indicated that coactivator presence and histone acetylation throughout the locus was stimulated to the same extent under both conditions, suggesting that the p21 expression may be 'primed' in both cases, but some later regulatory step controls the final outcome of p53 binding at the p21 locus. Interestingly, a subset of factors present only during sustained p21 expression was identified, consisting of TFIIB, TFIIF, and CDK8. This evidence suggests that, at least for a subset of p53 target genes, that the specific configuration of the transcriptional apparatus is tightly regulated, and itself dictates the transcriptional outcome of activator binding. Further research will be necessary to elucidate how, precisely, the recruitment of these factors to the p21 promoter is regulated.

Subsequent work from the Espinosa lab (Gomes and Espinosa, 2010b) unequivocally proves the model of p21 transcriptional activation cannot be extended to the exhaustive library of p53 target genes. High-resolution chIP and RNA analysis of the PUMA promoter revealed constant transcription, under basal conditions, of an unprocessed RNA of unknown function corresponding to the first 6 kb (first three exons) of the PUMA locus. Stimulation of PUMA expression by 5-fluorouracil (5-FU) effected little change in promoter occupancy of either p53, total RNAPII, phosphorylated RNAPII, or other assayed components of transcriptional complexes (in contrast to the p21 promoter, used for comparative purposes); yet transcription of the 6kb PUMA RNA was efficiently replaced by transcription with full-length PUMA mRNA. Strikingly, chromatin marks normally delineating gene boundaries(H3K4-me3) or transcriptionally accessible chromatin (H3K9-Ac) were all absent downstream of the third exon even after expression of wild-type PUMA induced by 5-FU, delineating what the authors refer to as

an “intragenic chromatin boundary” regulating transcriptional elongation. Importantly, the chromatin architecture of the PUMA locus appears to be unusual based on genome-wide analyses of chromatin marks throughout transcriptionally active chromatin (Guenther et al., 2007) – this example simply serves to further illustrate the complexity and heterogeneity of the p53 transcriptional response.

The most likely scenario, as is often the case in the gradual reconciliation of early reductionist perspectives, is that all of the models above make a partial contribution to the complex regulation of p53-dependent transcription. The repertoire of p53 target genes has changed and expanded throughout evolution, and these loci have evolved accordingly in different genomic regions at different times. The data seem to currently support the hypothesis that induction of p53 target genes is regulated at many steps after p53 binding for high-affinity promoters, whereas induction of low-affinity promoters seems to be generally regulated at steps following RNAPII binding. Additionally, evidence suggests that the architecture of the core promoter is dependent upon genomic context, and that the core promoter itself regulates kinetics of RNA expression in a largely activator-independent manner (Li et al., 2009). To fully explain the functional diversity of the p53 transcriptional response, continuing research dissecting the structural and functional elements of each p53-responsive promoter will be necessary.

In addition to the well-defined role of p53 as a transcriptional activator, a subset of genes containing p53 response elements are repressed by p53 (Riley et al., 2008). Transcriptional repression by p53 impacts a similarly broad scope of cellular processes as transcriptional activation, but are broadly consistent with the role of p53 as a tumor

suppressor; c-myc, surviving, cyclin B2, and the stem-cell marker Nanog are examples of growth-promoting or proto-oncogenic loci repressed by p53(Wang et al., 2010).

Precisely how an outcome of activation or repression by p53 is achieved on target promoters is an open question; however, some evidence suggests that the number, orientation, and sequence of p53 REs present in repressed genes may influence the outcome of p53-binding (Johnson et al., 2001; Murphy et al., 1999). Several analyses also suggest that spacer length may contribute significantly to p53 transactivation or repression potential – experimental alteration of spacer length has been shown to modulate both promoter occupancy and activation of p21 and surviving by p53 (Hoffman et al., 2002; Jordan et al., 2008); importantly, however, little correlation appears to exist between spacer length and activation versus repression when assayed on minimal promoters, suggesting that promoter context is critical in determining the transcriptional outcome of p53 binding.

p53 is believed to biochemically mediate transcriptional repression through one or more of several means – occlusion of *trans*-acting activator binding-sites by p53, recruitment of transcriptional corepressors such as histone deacetylases (HDACs), or squelching of transcriptional activators such as the TATA-box binding protein (TBP) (Seto et al., 1992). While some correlative evidence exists supporting these modes of regulation in p53-mediated repression, associations are far from absolute, implying that additional, as yet undetermined factors impact regulation of p53-mediated activation versus repression.

Transcription-independent Functions of p53

Mitochondrial Apoptosis

A well-characterized outcome of p53 activation in many contexts is apoptosis. Because p53 transactivates a multitude of pro-apoptotic genes, it was reasonably assumed that the apoptotic potential of p53 rested upon its activity as a transcription factor. In retrospect, however, it had been observed that p53 exhibits apoptotic activities in the presence of transcriptional inhibitors (Caelles et al., 1994) and, further, that transcriptionally-inactive p53 mutants maintain some apoptotic activity (Haupt et al., 1995). Mounting evidence suggests that p53 exerts its transcription-independent apoptotic activities through localization of a mono-ubiquitinated pool of p53 to the mitochondria where, through complexing with anti-apoptotic Bcl-2 family members through the p53 DNA-binding domain, it promotes the release of cytochrome C, thereby promoting apoptosis (Marchenko et al., 2007; Mihara et al., 2003). In hindsight, this function of p53 may serve as an additional explaination for the higher propensity of tumors to inactivate p53 through DNA-binding domain mutations rather than transactivation domain mutations.

DNA-damage repair

As in the case of apoptosis, p53 transactivates a wide array of genes contributing to DNA repair, but appears to play an additional direct role in transcription-coupled repair of some DNA lesions. A non-sequence-specific DNA-binding activity p53 likely serves to recruit chromatin remodeling factors such as p300 to sites of DNA-damage, relaxing

local chromatin conformation and allowing nucleotide excision repair (NER) factors to access damaged DNA (Rubbi and Milner, 2003). Another explanation not mutually exclusive with direct DNA-binding of p53 to sites of damage is that p53 associates with two subunits of the NER helicase TFIIH, XPB and XPD, at sites of DNA damage to direct accessibility of repair factors. p53 inhibits the helicase activity of TFIIH through direct interaction with the XPB helicase domain, potentially allowing the DNA-associated complex to 'lock' into a state where it can nucleate an active NER complex and allow repair to proceed (Wang et al., 1995).

Concluding Remarks

For over 30 years, p53 has been the subject of intense research, being found to have roles in nearly every facet of human physiology. Recent analyses suggest that p53 is the most mutated gene in human cancer, highlighting the critical role of its inactivation in tumorigenesis. How p53 coordinates the various processes that contribute to tumor suppression, and even the nature of the processes themselves, remains a topic of intense research and debate.

A piece of the puzzle critical to our understanding of p53, and transcription in general, is how sequence-specific transcription factors accurately regulate activation of specific sets of target promoters under specific conditions. While post-translational modifications of p53, and modulation of p53 complexes and stability play an important role in this selectivity, an additional level of complexity exists after p53 binding to its cognate sequences at the promoter itself. It will also be of critical importance to better

define how upstream activating signals such as DNA-damage or ARF result in the activation of a highly specific p53-dependent physiological program. The work presented herein aims to define the P/CAF acetyltransferase as a critical regulator of both p53 stabilization in response to p14ARF and, independently, a mediator of histone acetylation critical for p21 induction in response to a wide panel of p53-activating stresses.

Chapter II

The Histone Acetyltransferase P/CAF Regulates p21 Transcription Through Stress-induced H3K14 Acetylation

Abstract

Initiation of transcription by sequence-specific transcription factors is facilitated, in part, by one or more histone acetyltransferases (HATs). Several HATs, including p300, CBP, PCAF, and GCN5 have been implicated in the activation of p53-dependent transcription. Here we show that P/CAF is a critical regulator of p53-dependent p21 expression in response to a wide variety of genotoxic stresses. P/CAF was required for the transactivation of p21 expression by exogenous p53 in p53-null cells. P/CAF was also critical for p21 expression induced by nutlin-3, DNA-damaging agents, and p14ARF expression; suggesting a role for P/CAF in control of p21 expression in response to a wide range of cellular stresses. Surprisingly, transcriptional activation of p21 by the p53 K320R mutant was also dependent on P/CAF, suggesting that the role for P/CAF in p21 induction is independent of p53 K320 acetylation, a previously suggested target of PCAF-mediated acetylation. ChIP analysis of the p21 promoter also indicated that promoter occupancy by p53 was not altered by P/CAF knockdown, but that acetylation of H3K14 stimulated by p53 in H1299 cells was dependent upon P/CAF. Together our experiments indicate that P/CAF is required for full p53-directed transcription of p21 in part through regulation of stress-responsive histone 3 acetylation at the p21 promoter.

Introduction

Cellular responses to aberrant activation of proto-oncogenes in metazoans constitute a vital defense against neoplastic transformation, and are frequently lost or deregulated in cancer. The tumor suppressor p53 is mutated in about half of all cancers and is believed to be functionally antagonized in most or all cancers retaining wild-type p53. Tumor suppressive activities of p53 are achieved through both transcription-dependent and independent coordination of an array of cellular responses to various genotoxic stresses. Among the well-characterized transcriptional targets of p53 is the CDK-inhibitory protein p21, which is upregulated, often in a p53-dependent manner, in response to many physiologic stresses and results in a transient or sustained cell-cycle arrest. More recently, the p53-p21 signaling circuit has been shown to act as a barrier to both the generation of induced pluripotent stem (iPS) cells (Banito et al., 2009; Hong et al., 2009; Li et al., 2009), and tissue regeneration in mice (Bedelbaeva et al., 2010), highlighting the importance of developing a complete understanding of p21 regulation.

Histone/protein acetyltransferases (HATs) play a vital role in regulating transcription by acetylating histone tails, which in turn is believed to negatively regulate histone-nonhistone interactions and enhance chromatin accessibility of other transcription factors and adaptors. Additionally, many HATs directly regulate stability or activity of transcription factors, such as p53, through acetylation. Perhaps unsurprisingly then, some histone acetyltransferases (HATs) also harbor tumor suppressor activity and represent a promising class of therapeutic targets.

Among HATs known to act on p53 and enhance its transcriptional activity is the

p300/CBP-associated factor P/CAF (Di Stefano et al., 2005a; Liu et al., 1999). A member of the GNAT (GCN5-related N-acetyltransferase) family of protein acetyltransferases, P/CAF was originally identified as a factor displaced from p300/CBP-containing complexes after expression of the adenoviral oncoprotein E1A (Yang et al., 1996). The P/CAF complex is known to acetylate histones and various transcriptional regulators (Ogryzko et al., 1998), and has been shown to modulate activities of several tumor suppressors and oncogenes. Functional and structural characterization of P/CAF acetyltransferase activity indicates that K14 of histone H3 is the preferred substrate for this activity, and this residue appears to play a critical role in promoting the association of histone H3 with P/CAF (Poux and Marmorstein, 2003; Schiltz et al., 1999).

A direct role for P/CAF in p53 signaling has been previously suggested but is incompletely understood. Several reports using *in vitro* and overexpression analyses suggest that P/CAF is capable of directly acetylating p53 at lysine 320 and enhancing its site-specific DNA-binding activity, and that acetylation of this site increases after UV irradiation (Le Cam et al., 2006; Liu et al., 1999). Mass spectrometric analysis of p53 fragments acetylated *in vitro* by p300 and PCAF indicates that lysine-320 is indeed an *in vitro* substrate of P/CAF acetyltransferase activity (Sakaguchi et al., 1998). Several studies also utilize p53 acetyl-lysine specific antibodies to detect acetylated forms of p53 under various conditions, but cross-reactivity of these antibodies with other residues is a common caveat of this method. Therefore, *in vivo* and cell culture models describing the nuanced functions of P/CAF in p53 acetylation and activity are lacking.

Here we show that, under a variety of p53-activating stress conditions and cell

types, P/CAF is critical for p21 expression and p53-dependent cell-cycle arrest. This role of P/CAF in p21 expression is transcriptional, as shown by a P/CAF-dependent increase in p21 transcripts in p53-transfected H1299 cells by real-time rtPCR. Ablation of P/CAF by siRNA is associated with a loss of acetylation of H3K14, a previously described P/CAF-directed modification commonly associated with transcriptional activation, at the distal p53 response element of the p21 promoter. Levels of total RNA polymerase II loaded at the proximal promoter are correspondingly lower in the absence of P/CAF; furthermore, RNA polymerase II levels downstream of the proximal promoter correlate with the expression status of p21. These data indicate that P/CAF regulates p53-dependent p21 activation through histone acetylation at the p21 promoter, which creates an environment permissive for p21 transcription. Additional experiments will be required to determine which, if any, other p53-responsive promoters require P/CAF for their transcriptional activation.

Results

Two reports (Mellert et al., 2007; Sekaric et al., 2007a) indicate that p53 is acetylated in response to p14ARF expression in both the DNA-binding domain and the p53 C-terminus. To address the role of known p53 coactivators in p14ARF-directed p53 acetylation and activation, we surveyed the roles of p300, CBP, and P/CAF by siRNA-mediated depletion in U2OS cells. In line with previous reports (Grossman et al., 1993; Shi et al., 2009a), p300 and CBP appeared to negatively regulate p53 stability under basal conditions, presumably through their roles as E4 ubiquitin ligases (ubiquitin ligases

which recognize already mono-ubiquitinated proteins as substrates); strikingly, however, P/CAF depletion completely prevented stabilization of p53 and induction of p21 in response to ARF (Figure 2.1A). To rule out the possibility that this result was cell-type specific, we repeated the experiment in hTERT-immortalized, diploid RPE1 cells; again, P/CAF was necessary for ARF-directed p53 stablization and p21 induction (Figure 2.1B). To determine the effects of P/CAF depletion on the cell-cycle distribution of ARF-transfected U2OS or RPE1 cells, cell populations were stained with propidium iodide and analyzed by flow cytometry (Figure 2.2). As expected, p14ARF transfection induced a potent cell-cycle arrest in both U2OS and RPE1 cells; however, in P/CAF-depleted populations, p14ARF failed to induce a cell-cycle arrest.

To determine whether the involvement of P/CAF in p53 activity is specific to ARF, we examined the p53 and p21 response to a variety of p53-activating stresses in the presence or absence of P/CAF. First, we noticed that depletion of P/CAF by siRNA in p53-transfected H1299 cells resulted in a dramatic reduction in p21 levels (Figure 2.3A). We also tested the impact of P/CAF knockdown on a variety of stresses which induce p21 in a p53-dependent manner. Use of nutlin-3, a nanomolar competitive inhibitor of the MDM2-p53 interaction, which stabilizes endogenous p53 in U2OS cells, is known to result in induction of p21 (Vassilev et al., 2004); as expected, both p53 and p21 levels increased in response to nutlin-3 – however, while nutlin-3 also resulted in p53 stabilization in the absence of P/CAF, no corresponding increase in p21 levels was observed (Figure 2.3B). To ensure that the inability of this stress to culminate in p21 induction did, in fact, impact the cell-cycle distribution of these populations, we again

examined PI-stained cell populations by flow cytometry. While nutlin-3 treatment of U2OS cells resulted in a cell-cycle arrest, the P/CAF-depleted population again failed to undergo a comparable arrest (Figure 2.3E).

To observe a more physiologically relevant activator of p53 stress responses, we turned to ultraviolet radiation and the DNA-damaging agent Doxorubicin, which causes the accumulation of double-strand breaks and potently activates p53 signaling. After exposure to 100nM Doxorubicin for 24 hours (Figure 2.3C) or 20 J/m^2 UV (Figure 2.3D), control-siRNA expressing U2OS cells exhibited increased p21 levels, whereas P/CAF siRNA-expressing cells did not, despite a similar increase in p53 levels in both control or P/CAF-siRNA expressing cells.

To determine whether the loss of p21 induction led to changes in cell-cycle distribution, we examined DNA content of PI-stained cell populations of ARF-transfected or nutlin-treated cells using flow cytometry. In both ARF-expressing U2OS and RPE1 cells expressing control siRNA, the population showed a striking absence of S-phase cells, indicating a potent cell-cycle arrest; whereas these ARF-expressing populations failed to undergo cell-cycle arrest in the absence of P/CAF due to a loss of p21 (Figure 2.2A). Similar results were seen with nutlin-3 treatment, after which cells depleted for P/CAF failed to undergo a cell-cycle arrest (Figure 2.3E).

P/CAF is known to function as a transcriptional coactivator for several factors through its ability to acetylate histone and non-histone factors; however, P/CAF has also been recently shown to exhibit intrinsic E3 ligase activity toward MDM2 (Linares et al., 2007). An examination of steady-state levels of MDM2 and p53 in P/CAF-depleted

U2OS cells revealed no appreciable change in the levels of p53 or MDM2 (Figure 2.7). Therefore we wished to investigate whether p21 mRNA expression levels were being regulated by P/CAF. We performed quantitative rtPCR on H1299 cells transfected with a vector or wild-type p53 in combination with either control or P/CAF siRNA. Results of this experiment indicate that p21 mRNA is indeed upregulated following transfection of p53, but p21 transcripts remain unchanged in P/CAF siRNA-expressing cells as compared to the vector control, consistent with P/CAF exerting control over p21 expression through a transcriptional mechanism. Importantly, transcription of transfected p53 and endogenous β-actin are both unaffected by the absence of P/CAF (Figure 2.4), indicating that the impact of P/CAF knockdown on p21 expression is not a consequence of global effects on general transcription.

Because P/CAF has a previously described role in acetylation of p53 K320, we tested whether a p53 K319-321R mutant is competent for transactivation of p21 and, if so, whether P/CAF is also critical for p21 expression in this context. We found that p53 K319-321R stimulates p21 expression to a level comparable to wild-type p53, and that P/CAF is indeed critical for p21 expression driven by p53 K319-321R (Figure 2.6). These results indicate that, in addition to acetylation of p53 K320, P/CAF plays an additional, and critical, role in p53-dependent p21 expression.

Because P/CAF ablation modulates p21 transcription by both wild-type and K319-321R p53, we reasoned that P/CAF may be functioning through its histone acetyltransferase activity to regulate p21 transcription. P/CAF exhibits well-characterized HAT activity toward several lysines of histone 3, most potently lysine 14 (H3K14)

(Marmorstein and Berger, 2001) and, by global chIP-seq studies in wild-type and P/CAF-null MEFs, lysine 9 (Jin et al., 2011). To determine whether H3K14 acetylation in response to p21-inducing stimuli (p14ARF expression in U2OS cells or transfected p53 in H1299 cells) is impacted by loss of P/CAF, we analyzed by chromatin immunoprecipitation (chIP) the occupancy of the distal p53-response element of the p21 promoter by acetylated H3K14. In both ARF-transfected U2OS cells (Figure 2.5A) and p53-transfected H1299 cells (Figure 2.5B), H3K14 acetylation is stimulated in relation to untransfected cells; however, cells expressing P/CAF siRNA exhibit only baseline levels of H3K14 acetylation, which correlate closely with p21 expression status in our experiments.

We further reasoned that if P/CAF directly acetylates H3K14 to modulate p21 expression, and is not indirectly facilitating binding of other HATs to the promoter, this should be dependent upon P/CAF HAT activity. Using an internal deletion mutant of P/CAF deficient in HAT activity (Jiang et al., 1999), we introduced silent mutations into the siRNA-hybridizing region of the P/CAF ORF and rescued P/CAF knockdown with either a wild-type or HAT-deficient P/CAF allele. As expected, in H1299 cells expressing exogenous p53, co-expression of P/CAF siRNA with wild-type P/CAF restored p21 expression, whereas co-expression of P/CAF siRNA with the HAT mutant did not (Figure 2.5C). This experiment clearly indicates that P/CAF HAT activity is necessary for p21 expression in response to stress.

Figure 2.1: P/CAF is necessary for expression of p53 and induction of p21 in response to p14ARF in U2OS and RPE1 cells.

1X10^6 U2OS (Figure 2.1A) or RPE1 (Figure 2.1B) cells were plated and transfected with Lipofectamine 2000 after 24h with 10nM indicated siRNA and 1ug pcDNA3-p14ARF or pcDNA3 vector with 1ug salmon-sperm DNA (1.6ug DNA : 1uL Lipofectamine) and harvested 48h post-transfection. Cells were lysed in SDS lysis buffer (50mM Tris pH 6.8, 2% SDS) for SDS-PAGE analysis (Fig. 1A-1B). Cells were harvested 48hr post-transfection and lysed in 300-500uL 2% SDS with 50mM Tris pH6.8. Protein concentrations were determined using the BCA assay kit, 20ug protein loaded in each well and separated by SDS -PAGE.

Figure 2.1: P/CAF is necessary for expression of p53 and induction of p21 in response to p14ARF in U2OS and RPE1 cells.

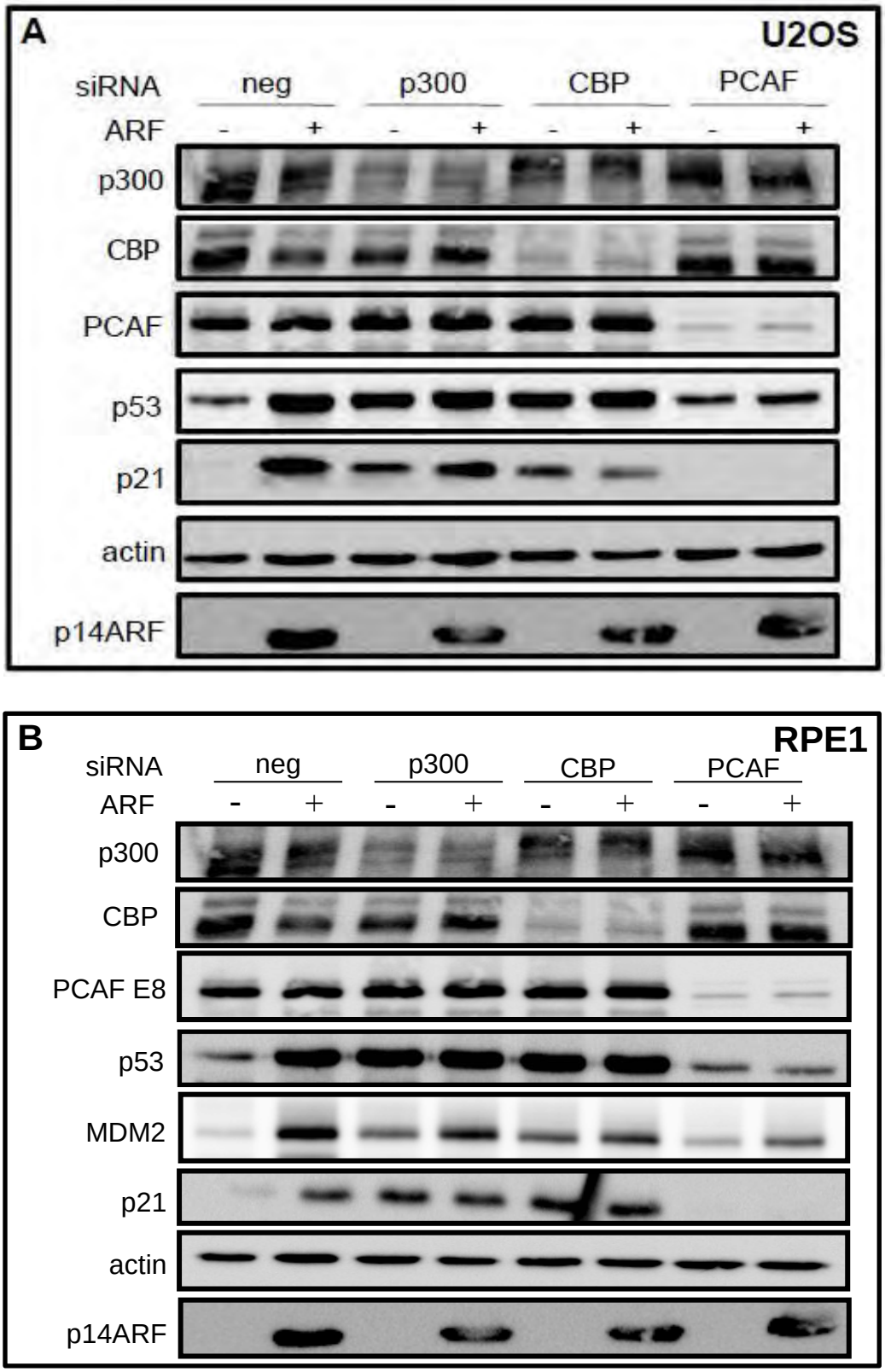


Figure 2.2: P/CAF is necessary for ARF-induced cell-cycle arrest in U2OS and RPE1 cells.

1X10^6 U2OS (Figure 2.2A) or RPE1 (Figure 2.2B) cells were plated and transfected with Lipofectamine 2000 after 24h with 10nM indicated siRNA and 1ug pcDNA3-p14ARF or pcDNA3 vector with 1ug salmon-sperm DNA (1.6ug DNA : 1uL Lipofectamine) and harvested by scraping 48h post-transfection. After 3 5mL PBS washes the cell pellet was fixed in ice-cold EtOH, stained with propidium iodide 48h later for flow cytometric analysis. 20,000 events were taken, including any sub-G1 population.

Figure 2.2: P/CAF is necessary for ARF-induced cell-cycle arrest in U2OS and RPE1 cells.

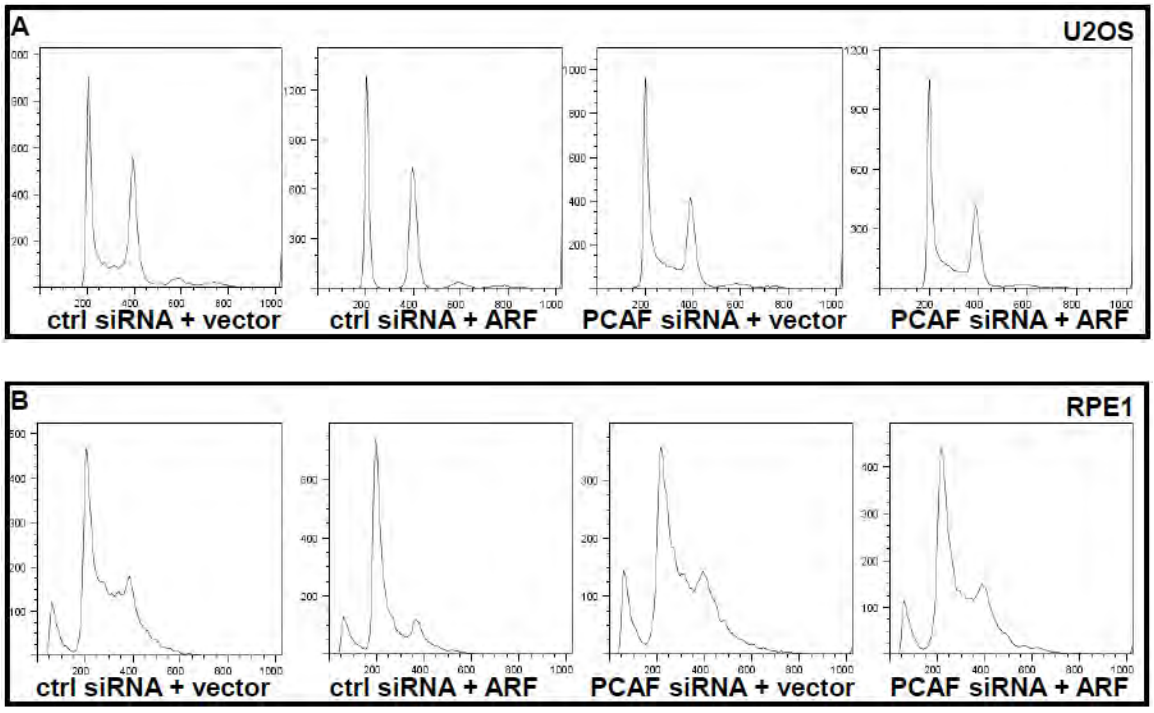


Figure 2.3: PCAF is required for p53-dependent p21 expression in a cell and stress-autonomous manner.

U2OS cells were plated at a density of 4x10^5 per 60mm plate or 1x10^6 per 100mm plate. H1299 cells were plated at a density of 6x10^5 per 60mm plate or 1.5x10^6 per 100mm plate. Cells were transfected and harvested for SDS-PAGE or flow cytometry as described above. Nutlin-3-treated U2OS cells were exposed to 5uM nutlin-3 for 16 hours prior to lysis or fixation. Doxorubicin-treated U2OS cells were exposed to 1uM Doxorubicin for 16 hours prior to lysis. UV-irradiated U2OS cells were dosed with 20 J/m^2 UVC prior to lysis using a Stratagene Stratalinker.

Figure 2.3: PCAF is required for p53-dependent p21 expression in a cell and stress-autonomous manner

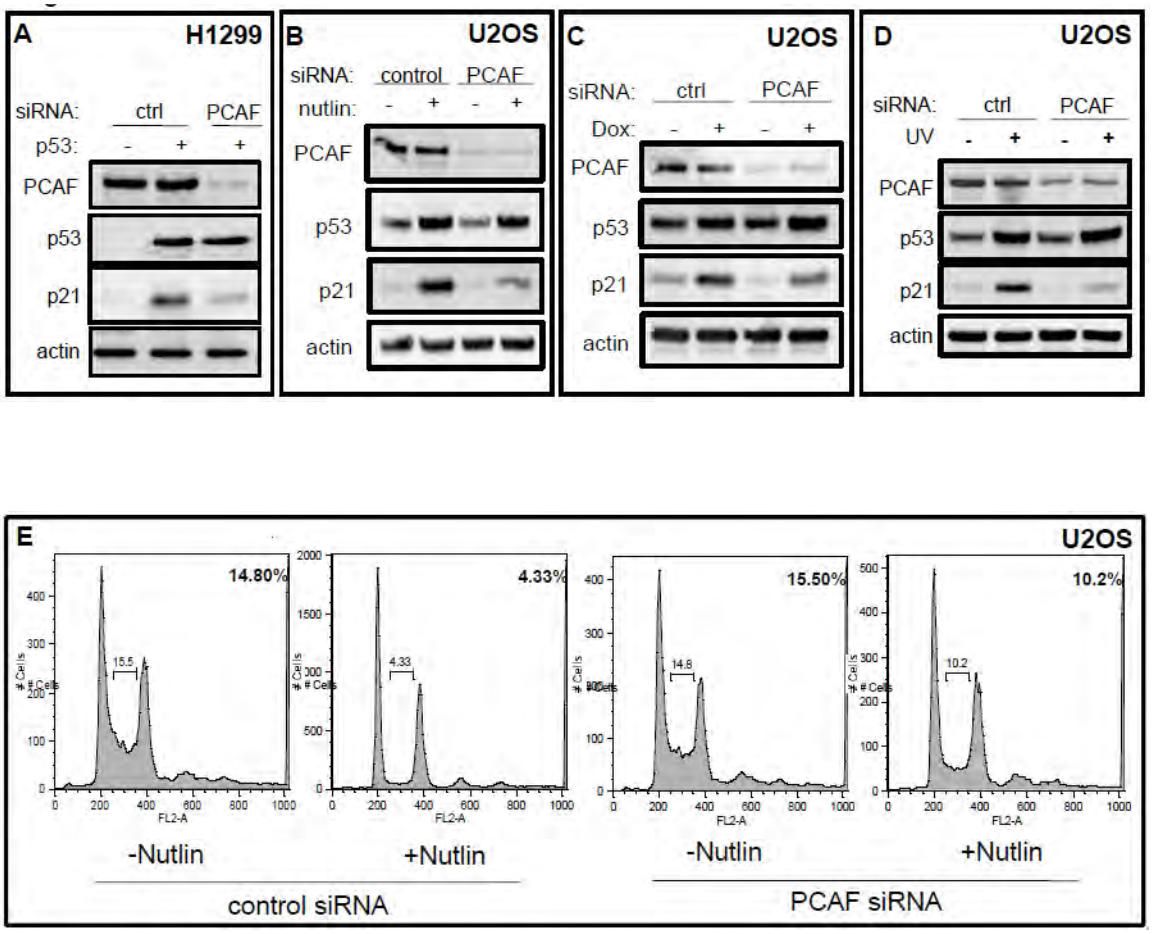


Figure 2.4: P/CAF is a transcriptional regulator of p21 expression.

H1299 cells were plated and transfected as described in previous figures, and total cellular RNA was purified using RNeasy kits (Qiagen). cDNA was generated using AffinityScript reverse transcriptase (Agilent Technologies). 1uL cDNA was amplified in triplicate using SYBR green and primers specific to β-actin, p21, or p53.

Figure 2.4: P/CAF is a transcriptional regulator of p21 expression

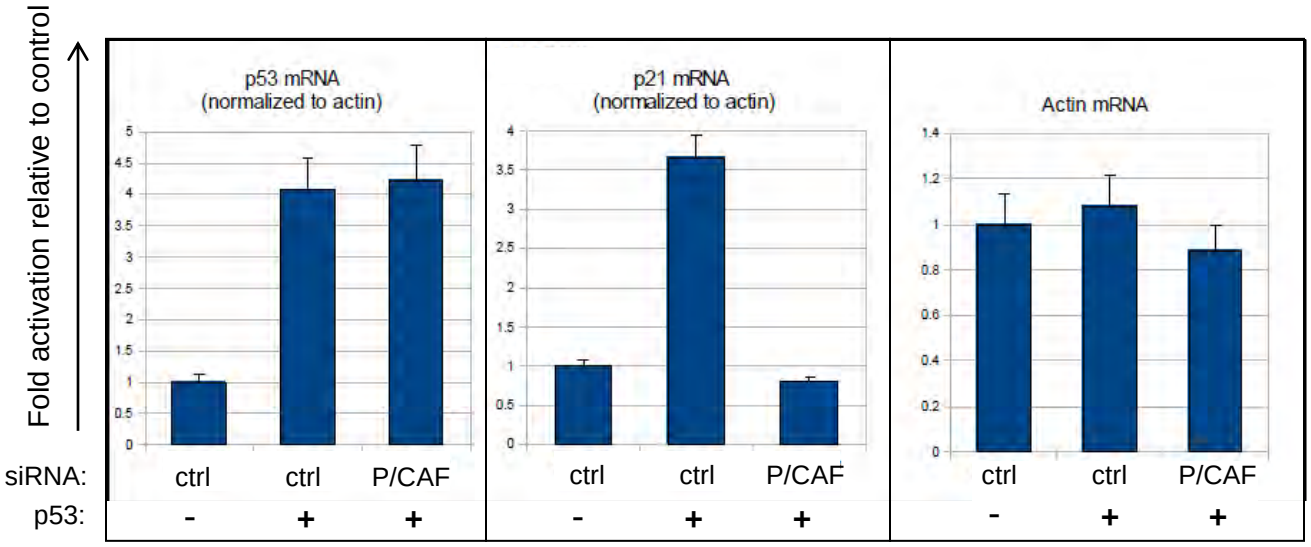
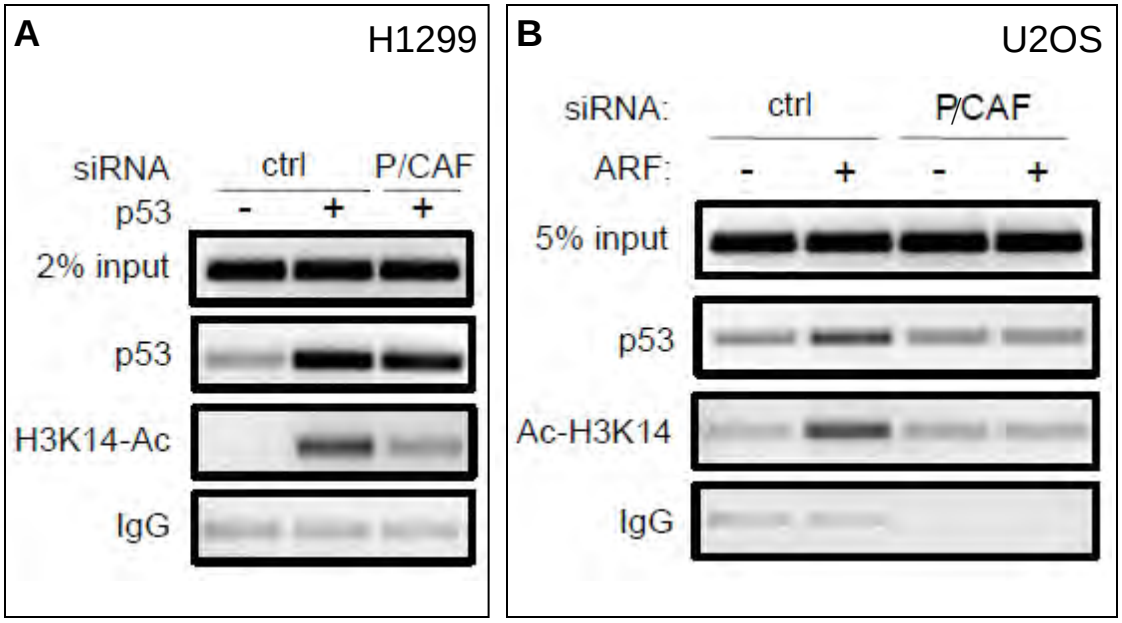


Figure 2.5: P/CAF regulates H3K14 acetylation at the p21 promoter.
A,B) Cells were plated and transfected as described above. Prior to lysis, cells were crosslinked with 1% formaldehyde for 10 minutes. Chromatin immunoprecipitations were performed as described in the Materials and Methods section. PCR was performed by amplifying the distal p53 response element of the p21 promoter with GoTaq polymerase (Roche) for 33 cycles for all samples. **C)** H1299 cells were transfected, lysed and separated by SDS-PAGE as in Figure 2.1. ΔHAT was generated by using PCR-based mutagenesis to generate a pCMV-P/CAF plasmid harboring an in-frame internal deletion of amino acids 608-628, and four silent mutations in the siRNA-hybridizing region of the open reading frame.

Figure 2.5: P/CAF regulates H3K14 acetylation at the p21 promoter



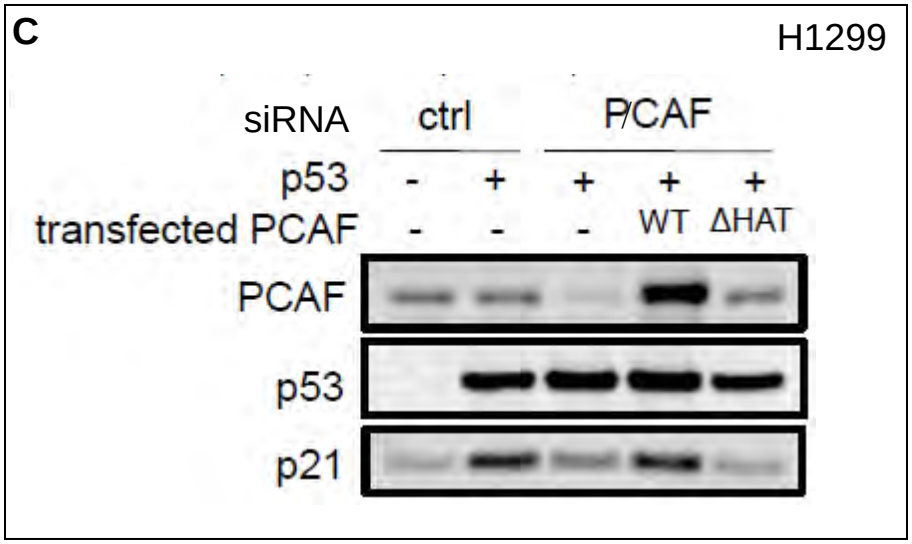


Figure 2.6: P/CAF regulates p21 expression independently of p53 K320 acetylation.

H1299 cells were transfected, lysed, and separated by SDS-PAGE as described above. WT indicates overexpression of a wild-type pCMV-p53 construct; 3KR denotes a pCMV-p53 construct in which lysines 319-321 have been mutated to arginine.

Figure 2.6: P/CAF regulates p21 expression independently of p53 K320 acetylation

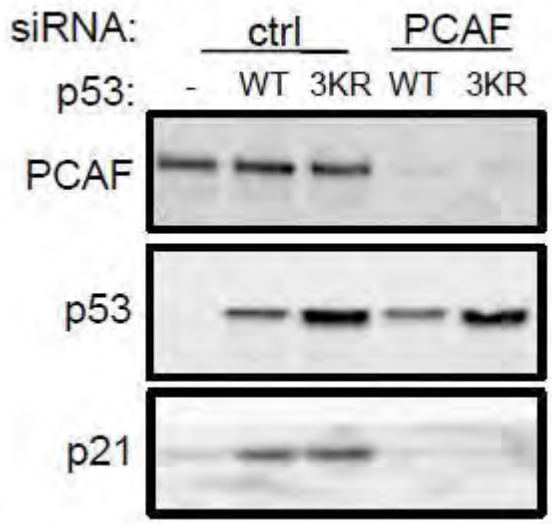


Figure 2.7: siRNA-mediated ablation of P/CAF does not alter basal levels of endogenous MDM2 or p53.

U2OS cells were transfected, lysed, and separated by SDS-PAGE as previously described. The three P/CAF siRNA sequenced used in this experiment are as follows:
PCAF-1: UCG CCG UGA AGA AAG CGC Att
PCAF-2: GGU GGU AUC UGU UUC CGU Att
PCAF-3: GGA GUC UUG UAA AUG UAA Utt

Figure 2.7: siRNA-mediated ablation of P/CAF does not alter basal levels of endogenous MDM2 or p53

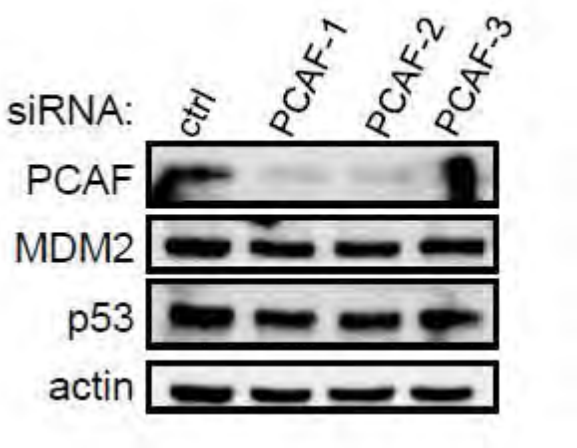
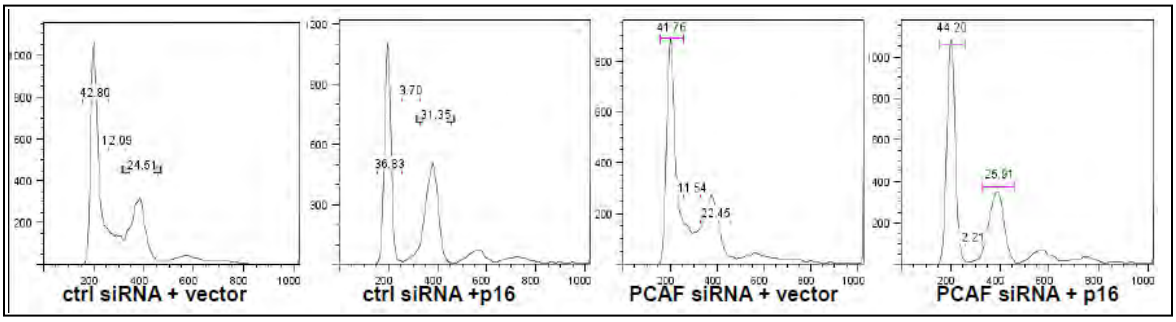
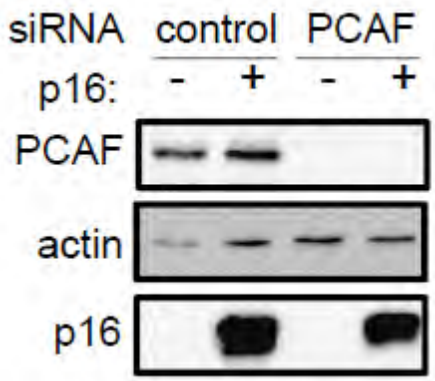


Figure 2.8: P/CAF Is Dispensable For p16-induced Arrest in U2OS Cells.

U2OS cells were transfected with 1ug pcDNA3-p16 or pcDNA3 with 4ug salmon-sperm DNA and 10nM indicated siRNA. Cells were harvested by scraping in PBS and washed 3 times with PBS. ¾ of the cell pellet was fixed in ice-cold 95% EtOH for at least 48 hours prior to PI staining and flow cytometry. The remaining ¼ of the cell pellet was lysed in SDS lysis buffer (2% SDS, 50mM Tris pH 6.8) for analysis by western blot.

Figure 2.8: P/CAF Is Dispensable For p16-induced Arrest in U2OS Cells





Discussion

Together, our data indicate that P/CAF is necessary to facilitate the stress-induced activation of the p21 promoter by creating a chromatin environment permissive for transcriptional activation. We have shown that P/CAF histone acetyltransferase activity is necessary for p53-directed p21 transcription in several cell types and in response to several stimuli. Loss of H3K14 acetylation at the p21 promoter is observed in the absence of P/CAF in p53-transfected H1299 cells, as well as ARF-transfected or nutlin-3 treated U2OS cells. This lack of histone acetylation correlates tightly with p21 expression status, mRNA levels. The impact of P/CAF on p21 expression requires an intact P/CAF HAT domain, suggesting that these effects are mediated by PCAF acetylase activity, and not simply through an interacting protein. This work defines a critical role for PCAF in p53-p21 signaling.

Significantly, these experiments provide a plausible explanation for previously described tumor suppressive functions of P/CAF. Despite the normal development of P/CAF-null mice (likely due to compensation by GCN5), P/CAF is downregulated through methylation or allelic loss in a variety of cancers (Perez et al., 2010; Zhu et al., 2009), notably, induces a G1 arrest and inhibits soft agar growth and tumorigenesis upon its reintroduction to cell lines established from these tumors. While the levels of several key cell-cycle regulators including p21 were evaluated in these studies, the mechanistic role of P/CAF in this activity was not explored further. Our data suggest that expression of p21 in response to a panel of genotoxic stresses specifically requires P/CAF HAT activity.

It has long been suggested that histone acetylation is permissive for transcriptional activation (Kuo and Allis, 1998; Sterner and Berger, 2000). However, mounting evidence indicates that histone acetylation at a promoter does not dictate the ultimate transcriptional status at a locus; indeed, several examples of histone acetylation at a transcriptionally silent p21 locus have been described (Donner et al., 2007; Gomes and Espinosa, 2010a); however, we are unaware of any reports in which p21 transcription proceeds in the absence of activating histone marks. Our data suggest a model in which recruitment of P/CAF HAT activity to the p21 locus by a transcriptional activator, in this case p53, allow for the occurence of subsequent downstream events that ultimately lead to elongation by the RNA pol II complex, which published works suggest is highly correlated with the transcriptional status of the p21 promoter. A report from the Espinosa lab (Donner et al., 2007) utilizing a high-resolution chIP for various factors at the p21 promoter under distinct stress conditions indicates that UV irradation of HCT116 +/+ p53 cells promotes accumulation of p53, acetylated histones, and certain general transcription factors at the promoter despite the gene remaining transcriptionally silent (and characterized by a lack of elongating pol II). In comparison, in nutlin-treated cells the accumulation of additional factors from the Mediator complex, as well as cyclin-C/CDK8 occurs along with elongating and serine-2 phosphorylated RNA pol II and, accordingly, p21 is expressed.

While we have clearly defined a novel role for P/CAF in p53-dependent p21 transcription through histone acetylation, the role of P/CAF in p53 signaling may be far broader. While we utilized a K319-321R p53 mutant to prove that P/CAF has additional

activities impacting p53, it does not rule out the possibility that under certain stress conditions or in specific cell contexts, K320 is a physiologically relevant acetylation target. Acetylation of either K320 or K382 is associated with occupancy of a unique set of p53-responsive promoters, interaction with a unique set of p53 coactivators, and differential roles in p53 localization (Knights et al., 2006). Additionally, one report suggests that P/CAF affects p53 signaling through modulation of human MDM2 (HDM2) stability by an E3 ubiquitin ligase activity intrinsic to P/CAF (Linares et al., 2007); however, using U2OS cells we observed no increase in steady-state MDM2 in PCAF-depleted cells (Figure 2.7). It is possible that P/CAF does harbor E3 ligase activity toward MDM2, but under the conditions in which these experiments were performed, P/CAF did not alter MDM2 levels. This raises the possibility that some additional culture stress(es) may be present in certain conditions which contribute to P/CAF E3 ligase activity toward MDM2.

Materials and Methods

Reagents

pCDNA-p53, pCDNA3-p14ARF, and pCDNA3 vectors were used in transfections. pCI-PCAF lacking the HAT domain (Δ608-629) and pCMV-p53 K319-321R were created by site-directed PCR mutagenesis, and both entire reading frames were sequenced to rule out the possibility of secondary mutations. Four silent mutations were introduced into the siRNA-hybridizing sequence to allow expression in the presence of siRNA. siRNA sequences used are as follows: SiRNA sequences: p300 – CAGAGCAGUCCUGGAUUACtt ; CBP - AAUCCACAGUACCGAGAAAUGUU ; PCAF – UCGCCGUGAAGAAAGCGCAtt ; PCAF-2 – GGUGGUAUCUGUUUCCGUAtt ; PCAF-3 – GGAGUC UUGUAAAUGUAAUtt

Cell Lines and Antibodies

RPE1, U2OS, and H1299 cells were grown under conditions suggested by ATCC. Antibodies used to detect endogenous protein levels were as follows: PCAF was detected with mouse monoclonal antibody E8 (Santa Cruz), p300 with rabbit polyclonal N-15 (Santa Cruz), CBP with rabbit polyclonal E14 (Santa Cruz) MDM2 with rabbit polyclonal antibody N20 (Santa Cruz), p53 with mouse monoclonal DO-1 or rabbit polyclonal FL-393 (Santa Cruz), p21 with mouse monoclonal 6B6 (Upstate), actin with rabbit polyclonal (Sigma), and acetylated H3K14 with (Active Motif #39599)

Transfections

RPE1 or U2OS cells were plated at a density of 1x10^6 cells / 10 cm dish 24 hours prior to transfection. H1299 cells were plated at a density of 1.5x10^6 cells / 10 cm dish 24 hours prior to transfection. Cells were then transfected with 10nM siRNA targeting p300, CBP, PCAF, or a negative control siRNA (Ambion) using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were transfected again with 10nM siRNA, 1ug pcDNA3-p14ARF or pcDNA3, and 4ug salmon-sperm DNA. Cells were harvested 48 hours after the second transfection, lysed using 300uL SDS buffer (2% SDS, 50mM Tris pH 6.8), boiled for 5 minutes and stored at -80C. Protein concentrations were determined using a BSA standard curve, and proteins were separated by SDS-PAGE and blotted using antibodies described above.

UV Irradiation, Nutlin-3, or Doxorubicin treatment

U2OS cells were plated and transfected as described above. Cells were treated with 5uM nutlin-3 for 16 hours prior to lysis/fixation, 20 J/m^2 UVC 24 hours prior to lysis/fixation, or exposed to 1uM Doxorubicin for a 16-hour duration prior to lysis/fixation.

Flow cytometry and PI staining

Cells were harvested 48 hours after second transfection by scraping and fixed in 75% EtOH for 24-72 hours at -20°C. Cells were stained with 50ug/mL propidium iodide in PBS and treated with 100uL of 100mg/mL RNAse prior to flow cytometric analysis by

the UMMS FACS core facility. Data was analyzed using FlowJo software (Tree Star, Inc.)

mRNA Isolation and Quantitative Real-time PCR

Total cellular RNA was isolated using RNAeasy kits (Qiagen) as described in the protocol. cDNA was then generated using AffinityScript reverse transcriptase (Agilent Technologies). 1uL cDNA was amplified in triplicate using SYBR green and primers specific to β-actin, p21, or p53. p53 and p21 signal was normalized to actin mRNA.

Chromatin immunoprecipitation

U2OS cells were plated into 8 15cm dishes at a density of 2.5x10^6 cells / 15cm dish. H1299 cells were plated into 8 15cm dishes at a density of 4x10^6 cells / 15cm dish. After transfections or nutlin-3 treatment as described above, cells were crosslinked with formaldehyde at a final concentration of 1% for 10 minutes, rotating at room temperature. Cells were washed 3 times with 15mL cold PBS after crosslinking. Cells were scraped ito 15mL conical tubes with 10mL PBS and centrifuged at 4,000rpm for 5 minutes. Nucleii were concentrated by lysing cell pellets in 10mL nuclear isolation buffer (5 mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40) for 20 minutes and centrifuging at 2,000rpm for 5 minutes. Nuclear pellets were lysed in 1mL nuclear lysis buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS) for 30 minutes on ice. Lysates were sonicated in an EtOH-ice bath for five 10-second pulses at an amplitude of 8 (out of 15). SDS was precipitated out by leaving on ice for 1 hour, supernatant was removed and DNA quantified at A260.

After diluting 1:1 in dilution buffer (16.7mM Tris pH 8.0, 167mM NaCl, 0.01% SDS, 1.1% Triton X-100), 150ug chromatin was pre-cleared for 1 hour at 4°C using 30uL Protein A and 30uL Protein G beads (Millipore) for each antibody used in each condition. Supernatant was removed after a 30 second spin at 1,000rpm, relevant chIP antibodies were added to each sample, and samples were rotated overnight at 4°C. 30uL Protein A and 30uL Protein G beads were added to each sample for 2 hours at 4°C to allow binding. Four 1mL high-salt washes (50mM HEPES pH 7.9, 500mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1 DOC) were performed, followed by two washes with TE buffer. TE buffer was aspirated, and beads were suspended in 300uL elution buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS) and 1uL Proteinase K (20ug/mL), and eluted for 1 hour in a 55°C heat block. Crosslinks were then reversed by incubating samples overnight in a 65°C heat block. DNA was purified using Qiagen PCR Purification columns and eluted in 50uL ddH2O. PCR was performed by amplifying the distal p53 response element of the p21 promoter with GoTaq polymerase (Roche) for 33 cycles for all samples.

Chapter III

Critical Requirement for P/CAF in p14ARF Signaling

Abstract

p14ARF activation has recently been shown to induce acetylation of p53 within both the DNA-binding domain and the C-terminal regulatory region. In an effort to identify the HAT or HATs which mediate this effect and to elucidate the importance of this modification in p14ARF signaling, we expressed specific siRNA oligos against multiple HATs implicated in the regulation of p53 function. In both human tumor cells and hTERT-immortalized RPE1 cells, siRNA-mediated ablation of p300 and CBP increased basal p53 levels and p21 activation and induced spontaneous cell cycle arrest while, surprisingly, knockdown of P/CAF prevented p14ARF-induced p53 and p21 expression, as described in Chapter II. P/CAF ablation also prevented cell cycle arrest after transfection of p14ARF. This effect was dependent upon the HAT domain of P/CAF, as a P/CAF mutant lacking the HAT domain was unable to rescue effects of P/CAF ablation on p53 activity. Surprisingly, siRNA-mediated reduction of p300, CBP, or P/CAF resulted in a loss of C-terminal acetylation at lysine-382 and phosphorylation of serine-15, indicating that these modifications are not absolutely required for p53 stabilization or activation, and further suggesting that the presence of all three HATs may be required for p53 phosphorylation and acetylation. Additionally, to rule out general effects of P/CAF knockdown on transcription and signaling, we demonstrate that p16-Rb

signaling is intact in the absence of P/CAF. These results were consistent in U2OS cells as well as non-transformed, hTERT-immortalized retinal pigment epithelial (RPE1) cells. To determine the manner in which P/CAF regulates p14ARF-dependent p53 stabilization, we performed co-immunoprecipitations of MDM2 with p14ARF and p53, and attempted to analyze the localization of these factors in the presence or absence of P/CAF. Co-immunoprecipitation of MDM2 with p14ARF and p53 were unaffected by P/CAF knockdown. Additionally, immunofluorescence experiments examining the localization of MDM2 in the presence or absence of P/CAF were inconclusive, but this possibility certainly warrants further investigation.

These experiments have defined the histone acetyltransferase activity of P/CAF as a critical and specific regulator of oncogenic stress signaling in human cells; in contrast, and in agreement with recent reports, p300 and CBP appear to play negative roles in p53 stability and activity under non-stress conditions. Ongoing work will seek to determine precisely by what mechanism P/CAF modulates p53 stability and activity in response to p14ARF.

Introduction

Surveillance of proto-oncogene activation is controlled in metazoans in large part through the products of the INK4A locus. In many cancers harboring wild-type p53, inactivation of the INK4A locus through hypermethylation is frequently observed. This locus encodes two potent yet structurally and functionally distinct tumor suppressors – p16INK4a and p14ARF. While p16INK4a inhibits Cdk4/6-mediated phosphorylation of

Retinoblastoma(Rb) through direct binding to Cdk4/6, p14ARF acts in the p53 pathway in part by blocking the E3 ligase activity of MDM2 toward p53, thereby stabilizing p53.

p14ARF also regulates p53 activity in part by modulating levels of acetylated p53 (Zhang et al., 2006). Following induction of p14ARF signaling (Sekaric et al., 2007a), p53 is acetylated on several C-terminal lysines in a manner dependent upon the transcriptional adaptor ADA3. A recent report also suggests that p53 is acetylated within the DNA binding domain following induction of p14ARF signaling. A lysine within the p53 DBD was recently described as a target of the MYST/Tip60 family acetyltransferases, but it is yet unclear what factor(s) mediate this effect in response to p14ARF. Most importantly, the physiological importance of these modifications in p14ARF signaling is unknown.

Transcriptional activation in eukaryotes is a highly complex and regulated process which is not completely understood. Eukaryotic chromatin structure presents a physical barrier to activation of transcription by regulating accessibility of the transcriptional machinery to specific loci. Transcription in response to various stimuli is often promoted by sequence-specific transcription factors which function to recruit coactivators, chromatin remodeling factors, and basal transcription factors to target promoters to facilitate transcriptional activation. Histone/protein acetyltransferases (HATs) play a vital role in regulating transcription by acetylating histone tails, which in turn is believed to positively or negatively regulate histone-nonhistone interactions and enhance accessibility of other transcription factors and adaptors, as well as regulate stability and activity of transcription factors through direct acetylation.

To date, p53 has been described as a substrate for several acetyltransferases; the coactivators p300 and CBP were first described to acetylate several C-terminal lysines of p53 and thereby enhance DNA-binding and transcriptional activation (Gu and Roeder, 1997; Lill et al., 1997). P/CAF, a member of the GNAT (GCN5-related N-acetyltransferase) family of protein acetyltransferases, was originally identified as a factor displaced from p300/CBP-containing complexes after expression of the adenoviral oncoprotein E1A. Purification of epitope-tagged P/CAF from HeLa cells identified several factors in the P/CAF complex including the transcriptional adaptor protein ADA3.

The role of P/CAF in p53 activity and stability is also incompletely understood. Several early reports using *in vitro* and overexpression analyses suggest that P/CAF is capable of directly acetylating p53 on lysine 320 and enhancing its site-specific DNA-binding activity, and that acetylation of this site increases after UV irradiation (Le Cam et al., 2006; Liu et al., 1999). Mass spectrometric analysis of p53 fragments acetylated *in vitro* by p300 and P/CAF indicates that lysine-320 is indeed an *in vitro* substrate of P/CAF acetyltransferase activity (Sakaguchi et al., 1998). Several studies also utilize acetyl-lysine-p53 specific antibodies to detect specifically acetylated forms of p53 under various conditions, but crossreactivity of these antibodies with other residues is a common caveat of this method. Therefore, *in vivo* and cell culture models describing the function of P/CAF in p53 acetylation and activity are lacking.

Here we present evidence that P/CAF is a critical and specific regulator of the ARF response in non-transformed human cells, whereas CBP and p300 are required to maintain a low level of latent steady-state p53 under basal conditions.

Results

P/CAF is required for p14ARF-induced p53 stabilization.

Because p53 acetylation is induced following p14ARF transfection, we reasoned that one or more HATs implicated in p53 activation is responsible for this effect. Several studies suggest that cellular HATs p300, CBP, and P/CAF play vital roles in p53 stability and activity. Therefore, siRNAs specifically targeting p300, CBP, or P/CAF were expressed both in U2OS cells and hTERT-immortalized RPE1 cells, then transfected with p14ARF or vector control. Surprisingly, ablation of CBP or p300 resulted in a drastic increase in basal p53 levels, suggesting negative roles for CBP and p300 in p53 stability consistent with previous reports; in contrast, ablation of P/CAF completely prevented stabilization of p53 after p14ARF transfection (Figure 2.1A-B).

P/CAF is required for p14ARF-induced p21 induction and cell cycle arrest.

To evaluate the biological effects of PCAF knockdown on p53 signaling, expression of two well-described transcriptional targets of p53 – p21 and MDM2 – was analyzed by western blot, and PI staining and flow cytometry were performed to examine cell cycle profile (Figure 2.2A-B). As expected, p21 and MDM2 expression closely correlated with p53 stabilization, indicating that stabilized p53 is capable of activating transcription under these conditions (Figure 2.1). Flow cytometric analysis of PI-stained populations indicates that p14ARF induces a potent cell cycle arrest, indicated by a complete loss of S-phase cells, which is prevented by PCAF knockdown (Figure 2.2A-

B).

Role of P/CAF in oncogenic signaling is specific to the p14ARF pathway and dispensable for p16INK4a-induced arrest.

P/CAF, p300, and CBP each possess a wide array of substrates for their histone acetyltransferase activity, suggesting the possibility that loss of these coactivators could have global effects on transcription which could affect a myriad of signaling pathways. To provide evidence that the role of P/CAF is specific to p14ARF signaling, we evaluated the role of P/CAF in p16INK4a-induced cell cycle arrest. Interestingly, ablation of P/CAF in both U2OS and RPE1 cells did not prevent cell cycle arrest in either cell line following transfection p16INK4a (Figure 2.8); however, p16INK4a-induced cell cycle arrest in cells expressing P/CAF siRNA displayed a greater propensity to undergo arrest in the G1 phase of the cell cycle, whereas G2 arrest predominated in cells expressing a control siRNA.

Loss of p53 K382 acetylation and S15 phosphorylation following HAT depletion

Because PCAF loss abolishes p53 signaling in response to p14ARF, we reasoned that a deficiency in PCAF-dependent acetylation could explain this effect. We probed lysates shown in Figure 2.1 with a monoclonal antibody specifically recognizing p53 acetylated at lysine-382, one of the C-terminal lysines of p53 predominantly acetylated in response to stress. We attempted to normalize the amount of total p53 between samples as detected by the polyclonal antibody FL-393 following densitometric analysis (data not

shown). As we expected, acetylation of lysine-382 was not detected in PCAF siRNA-treated samples following p14ARF transfection. Surprisingly, however, lysine-382 acetylation was also lost in p300 and CBP siRNA-treated samples despite the previously observed potent cell-cycle arrest and robust activation of p21 (Figure 3.1). These results indicate that acetylation of lysine-382 is not absolutely required for activation of p53.

N-terminal phosphorylation is known to enhance interaction of p53 with HATs (Barlev et al., 2001; Sakaguchi et al., 1998), and p14ARF has been reported to function through ATM/ATR to effect p53 activation (Li et al., 2004; Rocha et al., 2005). Based on these observations, and the observation that p14ARF promotes p53 acetylation (which is often associated with S15 phosphorylation), we wished to determine whether S15 is phosphorylated in response to p14ARF and, if so, whether this effect was dependent upon p300, CBP, or P/CAF. As expected, p14ARF strongly induced phosphorylation of S15 in both U2OS and RPE1 cells. Surprisingly, however, we noted that phosphorylation was attenuated by depletion of p300, CBP, or P/CAF. While residual phosphorylation was detectable after knockdown of p300 or CBP, none was detectable following P/CAF knockdown (Figure 3.2).

The MDM2 interaction with p14ARF or p53 is unaffected by P/CAF depletion

Because phosphorylation of S15 and acetylation of K382 was dependent upon the presence of P/CAF, we reasoned that disruption of the MDM2-p53 complex or formation of an MDM2-p14ARF may require P/CAF as an additional cofactor, and that its depletion may preclude efficient phosphorylation of MDM2 or p53. To address whether

P/CAF affects MDM2-p14ARF or MDM2-p53 complexes, we transfected p53-null H1299 cells, which maintain relatively high levels of p14ARF, with p53 and immunoprecipitated MDM2 in cells expressing either a control or P/CAF siRNA. As observed in previous experiments, P/CAF depletion had no impact on steady-state levels of MDM2, p53, or p14ARF, and MDM2-p14ARF and MDM2-p53 complexes appeared unaffected by P/CAF knockdown (Figure 3.3).

Analysis of MDM2 Localization in H1299 cells after P/CAF depletion

Much attention has been given to the observation that p14ARF appears to direct MDM2 to the nucleolus upon its expression (Weber et al., 1999), although the physiologic importance of this event is still contentious, and does not appear to constitute a strict requirement for p53 stabilization by p14ARF (Llanos et al., 2001). Because MDM2-ARF complex formation was unaffected by P/CAF depletion, we reasoned that the complex may fail to be properly localized to nucleoli after p14ARF expression, potentially allowing high levels of MDM2 to remain in the nucleoplasm and antagonize p53 activity. To address this possibility, we again utilized p53-transfected H1299 cells, as the steady-state levels of the proteins in question were previously observed to remain unchanged after P/CAF knockdown, allowing simpler interpretation of immunofluorescence experiments. Unfortunately, significant background staining for P/CAF was evident and, furthermore, we were unable to determine whether the intensity of MDM2 nucleolar staining was reduced upon depletion of P/CAF (Figure 3.4).

Figure 3.1: p300, CBP, and P/CAF are all necessary for acetylation of p53 K382 in response to p14ARF.

Plating, transfection, lysis, and separation by SDS-PAGE of U2OS and RPE cells was performed as described. 10ug each lysate was run in an initial experiment to determine total p53 levels (data not shown). Following densitometric analysis, lysates were separated again by SDS-PAGE with the purpose of loading equal amounts of total p53 to allow easier analysis of the fraction of K382-acetylated to total p53. Antibody specific to K382-acetylated p53 (#06-758) was purchased from Upstate.

Figure 3.1: p300, CBP, and P/CAF are all necessary for acetylation of p53 K382 in response to p14ARF

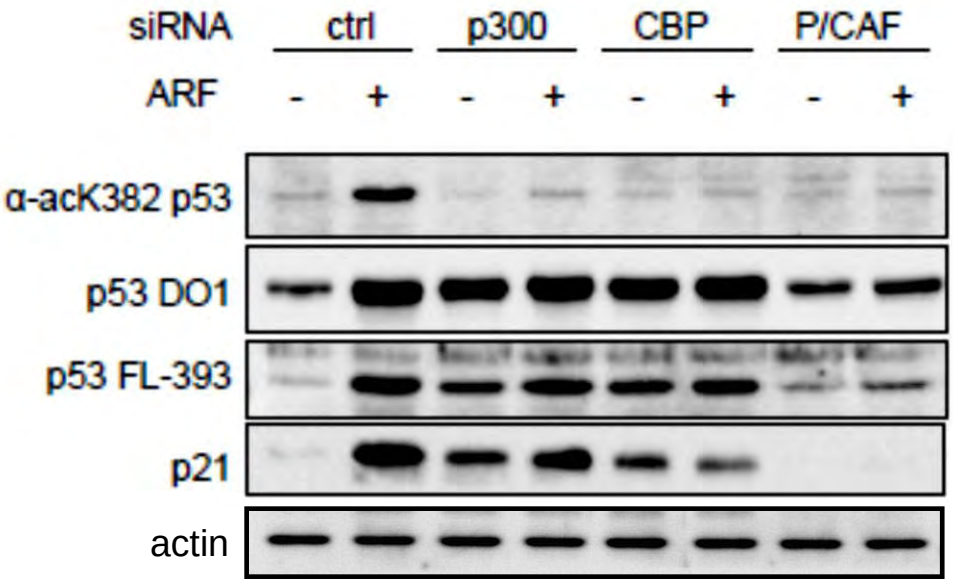


Figure 3.2: p53 serine-15 phosphorylation is induced by p14ARF and dependent on p300, CBP, and P/CAF.

U2OS and RPE1 cells were plated, transfected, lysed and separated by SDS-PAGE as described above. 20ug total protein was run from each sample, due to an inability to load enough lysate to equalize total p53 levels in this experiment. Antibody specific to S15-phosphorylated p53 (#9284) was purchased from Cell Signaling Technology.

Figure 3.2: p53 serine-15 phosphorylation induced by p14ARF in U2OS and RPE1 cells is dependent on p300, CBP, and P/CAF

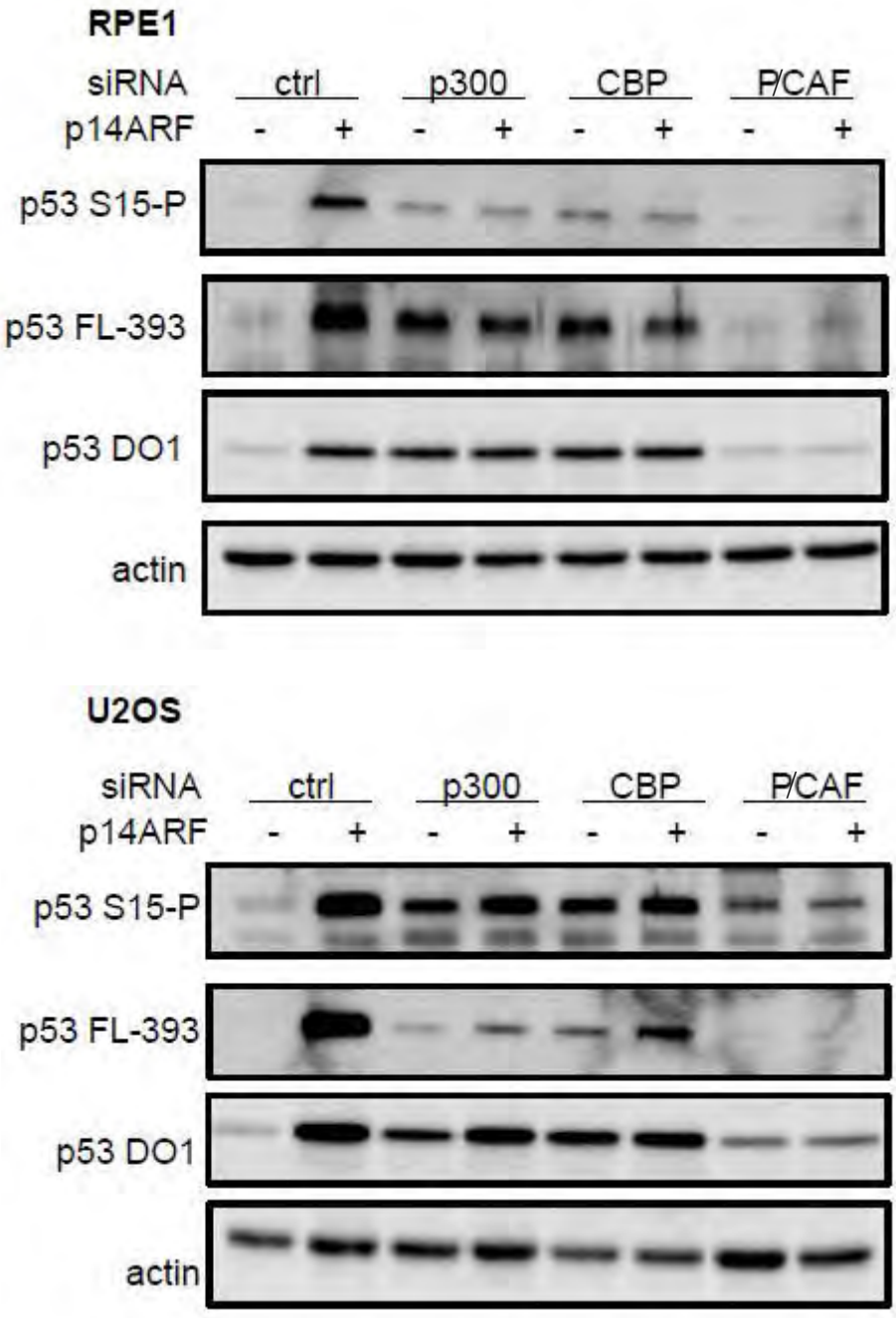


Figure 3.3: The MDM2 Interaction with p14ARF or p53 is Unaffected by P/CAF Depletion.
H1299 cells were plated at a density of 1.5x10^6 cells per 10cm dish, transfected with 10nM control or P/CAF siRNA using Lipofectamine 2000, and transfected the next day again with 1ug pCMV-p53 or pCMV using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours post-transfection with 1mL NP-40 buffer (1% NP-40, 150mM NaCl, 50mM Tris pH 8.0, 1mM PMSF, 100uM DTT) and lysed for 30 minutes on ice. Lysates were centrifuged at 12,000 rpm at 4°C, and 400uL each lysate was immunoprecipitated with 1ug rabbit IgG or MDM2 N-20 antibody (Santa Cruz) overnight. 20uL PBS-washed Protein A-agarose beads were added to each reaction and rotated for 1 hour. Reactions were then washed three times with 1mL NP-40 buffer and analyzed by SDS-PAGE. 20uL lysate was run on the same gel as a 5% input.

Figure 3.3: The MDM2 Interaction with p14ARF or p53 is Unaffected by P/CAF Depletion

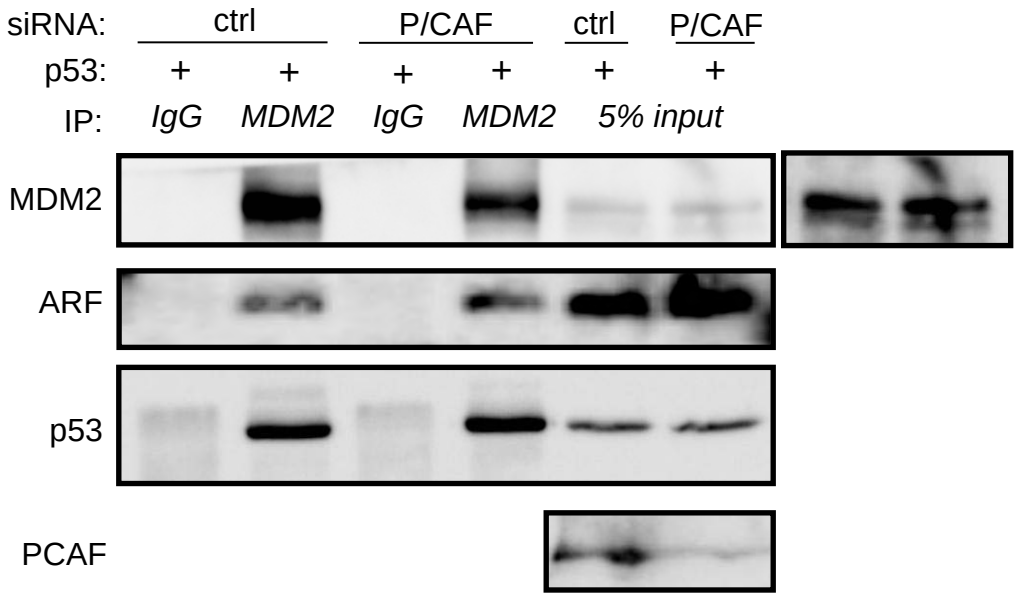
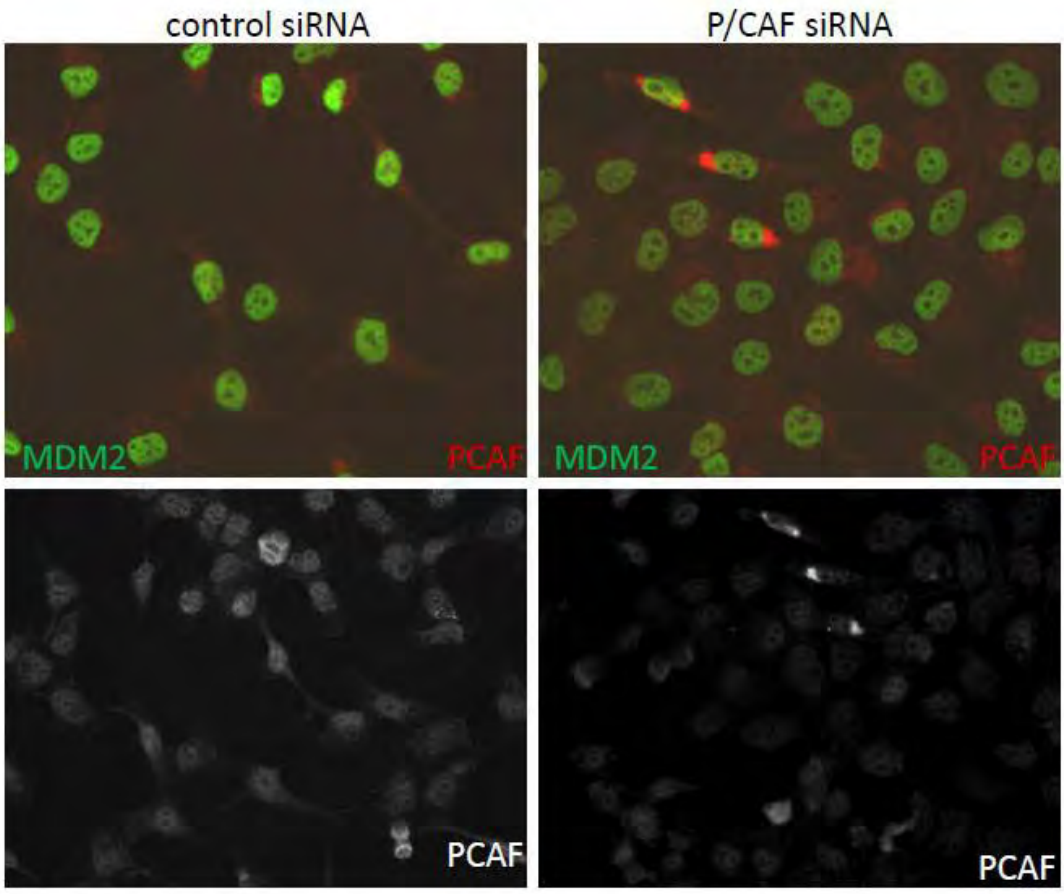


Figure 3.4: Analysis of MDM2 Localization in H1299 Cells After P/CAF Depletion. H1299 cells were plated on tissue culture dishes with an acid-washed cover slip at a density of 1x10^5 cells per 60mm dish. Cells were transfected the next day with 10nM control or P/CAF siRNA using Lipofectamine 2000 (Invitrogen), then again the next day with 10nM siRNA in combination with 200ng pCMV-p53. Cells were fixed in methanol 24 hours after transfection and stained with PCAF E-8 antibody (Santa Cruz) and MDM2 N-20 antibody (Santa Cruz). All imaging settings were set to the same parameters when imaging each population of cells.

Figure 3.4: Analysis of MDM2 Localization in H1299 Cells After P/CAF Depletion



Discussion

The p14ARF tumor suppressor is vital in transduction of oncogenic signaling to p53, which controls the context-dependent outcome of many cellular stresses. Ectopic p14ARF expression has been shown to induce acetylation of p53 on various lysines; however, the HAT or HATs which mediate acetylation of p53 in response to oncogenic stress remain unknown. Here we have demonstrated that p300/CBP-associated factor (P/CAF), but not p300 or CBP, is required for p14ARF-dependent stabilization of p53 and induction of p21 in human cells.

The Androphy lab recently reported that the human homolog of the transcription cofactor hADA3 is required for p14ARF signaling, and that siRNA-mediated loss of hADA3 or expression of a dominant negative N-terminal truncation abolishes p53 acetylation and stabilization in response to p14ARF, preventing p21 activation and cell cycle arrest (Sekaric et al., 2007b). Interestingly, hADA3 has been recognized as a component of the P/CAF complex (Li et al., 1998). Because loss of hADA3 or P/CAF function results in a similar loss of p14ARF signaling, it is likely that the activity of an intact P/CAF complex is required to transduce oncogenic signals from p14ARF to p53, and may act at the level of acetylation.

That p53 is acetylated in response to stress stimuli is well established; it was the first non-histone protein determined by mass spectrometric analysis to be acetylated on various lysines. However, the importance and impact of this modification on p53-dependent senescence, arrest, or apoptosis has not been well-studied. Most reports to date have utilized *in vitro* acetylation or overexpression assays to demonstrate that p53 is a

substrate of of many HATs.

The role of P/CAF in p53 stability and transcriptional activity is not well understood. Mechanistically P/CAF may activate p53 directly by acetylation of p53 protein or indirectly by modulating activities of other proteins involved in p53 regulation. Site-specific antibodies detect a very modest increase in Ac-K320 signal following UV irradiation, but the significance of this modification is unknown. Most reports that P/CAF affects p53 activity through acetylation utilize *in vitro* and overexpression studies, and detect acetylation using site-specific antibodies which are often unreliable, as they often cross-react with other residues or have off-target affinities. Although p53 and P/CAF have been reported to interact directly in GST-binding experiments (Liu et al., 1999), our attempts to co-immunoprecipitate FLAG-tagged P/CAF with p53 after overexpression in H1299 cells were not successful so far (data not shown). Mass spectrometry of *in vitro* acetylated p53 fragments does indicate that lysine-320 is substrate of P/CAF *in vitro* (Sakaguchi et al., 1998); however, the transcriptional activity of a p53 lysine-320 mutant is only mildly affected when assayed by a PG13-luciferase reporter. The experiments presented here highlight a much more critical role for P/CAF in p53 signaling, as its ablation by siRNA abolishes p14ARF signaling upstream of p53 (Figure 2.1)

P/CAF is known to be involved in MyoD-dependent differentiation through regulation of p21 induction (Puri et al., 1997); however, a role for P/CAF upstream of p53 stabilization is surprising. A recent report suggests that P/CAF bears E3 ubiquitin ligase activity toward MDM2 *in vitro* and *in vivo* (Linares et al., 2007), and modulation of MDM2 levels and activity presents an attractive model for stress-induced p53

stabilization; however in our experiments, both a widely-used monoclonal and polyclonal antibody against MDM2 failed to detect a significant change in MDM2 levels after P/CAF knockdown (Figure 2.7).

This does not rule out the possibility that P/CAF exerts other effects on MDM2 such as relocalization to the nucleolus; however, this function of ARF has been proposed to be dispensable for signaling to p53, at least in mice (Llanos et al., 2001). Interestingly, however, our data indicate that P/CAF may indeed play a role in the relocalization of MDM2 to the nucleolus (Figure 3.4). Many further experiments will be necessary to test this intriguing hypothesis. Most importantly, a necessary control that should be included in a repeat experiment is depletion of p14ARF by siRNA, which should result in potent nucleolar exclusion.

Interestingly, data from p53-null H1299 cells (Figure 2.6) indicates that P/CAF is required for activation of p21 downstream of p53 stabilization. When p53 is expressed in H1299 cells p21 is strongly induced, indicating an otherwise intact p53 signaling pathway; however, co-expression of P/CAF siRNA with p53 does not culminate in p21 induction, despite the presence of similar p53 levels. These data suggest that P/CAF is not only important for ARF signaling upstream of p53 stabilization, but that P/CAF is also required for p21 induction in the presence of high levels of p53. This may reflect a requirement for two independent functions of P/CAF; a well-described transcriptional co-activator function, necessary for initiating p53-dependent transcription at target promoters through histone acetylation, and a function in promoting p53 stabilization which has yet to be elucidated.

A negative role for p300 and CBP in regulation of steady-state p53 levels is surprising given that several reports suggest that p300 and CBP serve as coactivators for p53-dependent transcription (Lill et al., 1997; Scolnick et al., 1997). Interestingly, recent evidence suggests that p300 bears E3 ubiquitin-ligase activity *in vitro* toward p53 (Grossman et al., 2003a). In agreement with this study, our *in vivo* data demonstrate that loss of endogenous p300 or CBP result in an increase of steady-state p53 in the absence of any specific stress stimulus. A coactivator role for p300 in activation of p53-dependent transcription is clear, but how the newly described ubiquitin ligase activity of p300 impacts p53 signaling has not been explored. It is possible that coupled coactivation and ubiquitin ligase activities play vital roles in transcription; recent studies suggest that transcription factors may be 'cleared' off target promoters through the ubiquitin-proteasome system (Muratani and Tansey, 2003). We cannot rule out the possibility that loss of p300/CBP may increase p53 levels indirectly by induction of oxidative stress, which could result in activation of p53 signaling. As ATM was reported to be required for the p14ARF response (Li et al., 2004), it is possible that P/CAF is required for ATM signaling to p53 upon p14ARF expression.

That knockdown of p300/CBP induces accumulation of p53 is in agreement with several recent studies (Grossman et al., 2003b; Linares et al., 2007; Shi et al., 2009b). These data suggest a negative role of p300/CBP in p53 regulation. Paradoxically, the data also suggest that p300 and CBP are required for K382 acetylation (Figure 3.1), supporting a dual role for p300/CBP in p53 regulation. Moreover, p53 accumulation without acetylation of K382 is a surprising and unexpected observation that suggests an

alternative mechanism of p53 stabilization that does not involve K382 acetylation – perhaps primarily achieved through inhibition of p53 ubiquitination.

One intriguing possibility is that the presence or absence of P/CAF in the p300/CBP HAT complex constitutes a 'switch' in activity between a degrading or activating complex. Most endogenous MDM2 has been shown to be in complex with p300, and these complexes are known to play a role in p53 degradation (Grossman et al., 1998). Furthermore, adenoviral E1A displaces P/CAF from the p300/CBP complex and stabilizes p53 in a p300/CBP-dependent manner, suggesting that the presence of P/CAF in the complex could serve as a bias toward activation and against degradation of p53; furthermore, given that HPV E6 was recently shown to inhibit p300-mediated p53 acetylation in an *in vitro* system containing reconstituted chromatin templates (Thomas and Chiang, 2005) , it will be interesting to further investigate the role of viral oncogenes on various coactivator complexes known to play a vital role in tumor suppression.

Materials and Methods

Reagents

pCI-PCAF, pcDNA3-p14ARF and pcDNA3 were used for DNA transfections and have been previously described (Llanos et al., 2001). 10uM Trichostatin A (TSA) was added to lysates to be blotted with acetylation-specific antibodies. pCI-PCAF lacking the HAT domain (Δ608-629) was created by site-directed PCR mutagenesis and sequenced to rule out the possibility of secondary mutations.

Cell Lines and Antibodies

RPE1 cells and U2OS cells were grown under conditions suggested by ATCC. Antibodies used to detect endogenous protein levels were as follows: PCAF was detected with mouse monoclonal antibody E8 (Santa Cruz), p300 with rabbit polyclonal N-15 (Santa Cruz), CBP with rabbit polyclonal E14 (Santa Cruz) MDM2 with rabbit polyclonal antibody N20 (Santa Cruz), p53 with mouse monoclonal DO-1 or rabbit polyclonal FL-393 (Santa Cruz), p21 with mouse monoclonal 6B6 (Upstate), actin with rabbit polyclonal (Sigma), and Ac-K320 and Ac-K382 p53 with rabbit polyclonal antibodies (Upstate).

Transfections

RPE1 or U2OS cells were plated at a density of 1x10^6 cells/10cm dish 24 hours prior to transfection. Cells were then transfected with 10nM siRNA targeting p300, CBP, PCAF, or a negative control siRNA (Ambion) using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were transfected again with 10nM siRNA, 1ug pcDNA3-p14ARF or pcDNA3, and 4ug salmon-sperm DNA. Cells were harvested 48 hours after the second transfection, lysed using 300uL SDS buffer (2% SDS, 50mM Tris pH 6.8), boiled for 5 minutes and stored at -80C. Protein concentrations were determined using a BSA standard curve, and proteins were separated by SDS-PAGE and blotted using antibodies described above.

Flow cytometry and PI staining

Cells were harvested 48 hours after second transfection by scraping and fixed in 75% EtOH for at least 24 hours. Cells were stained with 50ug/mL propidium iodide in PBS and treated with 100uL of 100mg/mL RNAse prior to flow cytometric analysis by the UMMS FACS core facility. Data was analyzed using FlowJo (Tree Star, Inc.)

Immunoprecipitation

H1299 cells were plated at a density of 1.5x10^6 cells per 10cm dish, transfected with 10nM control or P/CAF siRNA using Lipofectamine 2000, and transfected the next day again with 1ug pCMV-p53 or pCMV using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours post-transfection with 1mL NP-40 buffer (1% NP-40, 150mM NaCl, 50mM Tris pH 8.0, 1mM PMSF, 100uM DTT) and lysed for 30 minutes on ice. Lysates were centrifuged at 12,000 rpm at 4°C, and 400uL each lysate was immunoprecipitated with 1ug rabbit IgG or MDM2 N-20 antibody (Santa Cruz) overnight. 20uL PBS-washed Protein A-agarose beads were added to each reaction and rotated for 1 hour. Reactions were then washed three times with 1mL NP-40 buffer and analyzed by SDS-PAGE. 20uL lysate was run on the same gel as a 5% input.

Chapter IV

Conclusions and Future Directions

The nature of biological research necessitates the continuous refinement of models and hypotheses as new information is brought to light, and the field of p53 transcription is no exception. Beginning, as responsible scientific inquiry does, with the simplest explanation which fits the existing data, the model of p53 transcriptional activation has ballooned into a strikingly complex one, whereby the cellular fate directed by p53 relies on countless levels of transcriptional regulation few would have ever suspected. Beyond the value of p53 as a model for the broad study of transcriptional processes, a complete understanding of a transcriptional network so central to human disease will someday allow us to manipulate these networks for eventual therapeutic benefit. In addition, an equally comprehensive understanding of the upstream signals which govern p53 activation will complement this knowledge, potentially allowing the restoration of these stress sensors in cells with defects in the p53 network through a number of means. The work presented in this thesis defines the histone acetyltransferase P/CAF as playing vital and independent roles in both p53 transactivation of p21 and transduction of oncogenic signaling from p14ARF to p53, and challenges its putative role in p53 activity as being confined to acetylation of p53 K320.

The Role of P/CAF in Stress-induced p21 Transcription

An siRNA-based approach was undertaken to examine the role of p300, CBP, and

P/CAF in p53 stabilization and p21 induction in response to p14ARF transfection in U2OS cells. It was noted that p300 and CBP depletion resulted in spontaneous elevation of p53 and p21 levels, consistent with a negative role for p300 and CBP in p53 stability and activity under basal conditions (Shi et al., 2009a). P/CAF, in contrast, prevented p53 stabilization and p21 induction after p14ARF transfection (Figure 2.1).

Reasoning that a defect in p53 acetylation in the absence of P/CAF might explain the failure in p53 stabilization and p21 induction, we turned to p53-null H1299 cells with which we could study various p53 mutants and their p21 response. Surprisingly, p21 expression induced by transfected, wild-type p53 was dependent on P/CAF in H1299 cells, despite p53 being present at similar levels in all conditions (Figure 2.3A). This result clearly indicates that P/CAF plays a role in p21 expression independently of regulating p53 levels. Reasoning that P/CAF was necessary for p21 expression through acetylation of p53 K320, we transfected a p53 K319-321R mutant into H1299 cells, expecting it to be deficient in inducing p21 expression; surprisingly, the K319-321R mutant was fully competent for p21 induction, which again was fully dependent upon the presence of P/CAF (Figure 2.6). This result clearly indicates that P/CAF plays a critical role in p53-dependent p21 expression independent of K320 acetylation.

Subsequent experiments in U2OS cells indicate that P/CAF is required for p21 induction in response to a variety of p53-activating stresses – UV irradiation, doxorubicin , nutlin-3, and p14ARF expression; interestingly, P/CAF knockdown did not affect stress-induced p53 accumulation in any case except p14ARF expression (Figure 2.3), suggesting a role for P/CAF in p53 stabilization specific to p14ARF, which will be

discussed in more detail below.

The work presented in Chapter 2 raises a number of questions which will be addressed by future experiments. Of immediate interest is whether the role for P/CAF in p53-activated transcription is limited to p21, or a broader subset of p53-responsive genes. One assumption challenged by recent work attempting to unravel the complexity of the p53 transcriptional response is that p53-responsive genes can simply be categorized by their roles in apoptosis or cell-cycle arrest, or by a simple division based on promoter affinity. The recent and thorough analyses of the PUMA and p21 promoters by several labs (Donner et al., 2007; Gomes and Espinosa, 2010b; Morachis et al., 2010) suggests that the native chromatin architecture inherent to specific promoters greatly impacts activation and expression kinetics. These recent observations preclude the prediction of which additional p53 targets might depend on P/CAF, but it is possible that P/CAF might be recruited to p53-responsive genes located in tightly or heavily chromatinized regions of the genome where H3K14 acetylation is required to allow de-compaction of chromatin to facilitate transcriptional initiation and elongation. Another possibility is that P/CAF might be recruited to those target genes which require numerous rounds of transcription to produce a sustained elevated expression level, such as p21. Regardless of whether such predictions may serve to be informative, a thorough analysis of known p53 target genes by chIP analysis of P/CAF is a necessary next step to more accurately determine the global role of P/CAF in p53-dependent transcription.

Another major question arising from this work is which p53 HAT or HATs mediate K320 acetylation. Results from previous experiments in the Androphy lab

(unpublished) using p53-transfected H1299 cells indicate that P/CAF knockdown actually increases basal levels of acetylated K320 and decreases levels of acetylated K382, while p300 overexpression in H1299 cells strongly stimulates K320 acetylation. It will be important to validate published results in this system by showing that, as published, P/CAF overexpression stimulates K320 acetylation. Several simple experiments could address the apparent contradiction that both P/CAF overexpression and ablation stimulate K320 acetylation. One possibility is that elevated GCN5 in P/CAF-depleted cells could promote K320 acetylation. This possibility is easily addressed through a simultaneous knockdown of P/CAF and GCN5. Another possibility is that P/CAF and/or GCN5 serve as adaptors for K320 acetylation by p300 or CBP. Several experiments could address this – first, a P/CAF or GCN5 HAT mutant should still stimulate K320 acetylation, assuming such a mutant could still serve as an adaptor for the relevant HAT. Another informative experiment would be overexpression of p300 or CBP in a P/CAF and GCN5-depleted population of cells. If p300 or CBP are K320 acetyltransferases dependent upon P/CAF or GCN5, they should stimulate acetylation of K320 only in the presence GCN5 or P/CAF. These experiments could address the confounding issue of which, if any, of these HATs are responsible for K320 acetylation. Identification of the HAT(s) mediating K320 acetylation will yield valuable insight into the regulation of cell fate by HATs, as acetylation of K320 seems to be an important factor in cell-fate decision by p53.

Intriguingly, our work indicates that p53 is pre-loaded at the p21 promoter, yet histone acetylation at the promoter increases in response to stress, raising the question of

how acetylation is stimulated. That the interruption of the p53-MDM2 complex alone by nutlin-3 is sufficient to promote p21 expression and histone acetylation at the promoter suggests that MDM2 may play a role in this process, perhaps through association with the p21 promoter in non-stress conditions. MDM2 is well-known to interact with HDAC1 to antagonize p53 function through deacetylation (Ito et al., 2002; Ito et al., 2001), raising a few possibilities: that MDM2-mediated deacetylation of p53 at the p21 promoter inhibits its interaction with HATs, or that a relevant function of the MDM2-HDAC1 may, in fact, be deacetylation of histones at p53-responsive promoters. Detection of P/CAF at the p21 promoter or in complex with p53 has so far been problematic, despite reports describing both promoter and direct interactions (Dell'orso et al., 2011; Knights et al., 2006). A thorough chIP analysis of the dynamics of p53 complexes present at the p21 promoter before and after stress should begin to address how histone acetylation at the p21 promoter is regulated.

Finally, the results presented herein further support a critical role for histone acetylation at the p21 promoter in p21 transcriptional activation. Work from the Espinosa lab indicate that levels of H3K9 and total H4 acetylation at the p21 promoter increase under stress conditions, even those that do not culminate in p21 transcription (Donner et al., 2007), suggesting that subsequent regulatory events define the final outcome. Our work complements this view, suggesting that, while histone acetylation at the p21 promoter is not sufficient to direct transcriptional activation, it may be necessary for additional regulated downstream which promote elongation of the RNAP II complex. While histone acetylation at other p53 target promoters is likely important for their

activation, the factors necessary for transcriptional activation may differ between p53 target promoters.

The Role of P/CAF in the p14ARF-p53 Oncogenic Stress Response

The second body of work in this dissertation characterizes the role of previously identified p53 HATs in p14ARF-p53 signaling. A better understanding of how p14ARF transduces oncogenic signals to direct cell-cycle arrest or senescence will be critical to the identification of therapeutically relevant targets. Furthermore, p14ARF signaling has found renewed importance with the recent observation that p14ARF-p53 signaling is a barrier to the generation of induced pluripotent stem (iPS) cells (Banito et al., 2009; Hong et al., 2009; Li et al., 2009). Identification and characterization of factors regulating p14ARF signaling might potentially be useful in the short-term inactivation of this pathway to facilitate reprogramming.

This project began with the same siRNA-based approach as described in the previous section. In this experimental setting, p300 and CBP played negative roles in p53 stability and activity, as their abrogation resulted in spontaneous stabilization and activation of p53, as evidenced by increased basal p21 levels. The results were virtually indistinguishable in the presence of p14ARF. Because p300 and CBP are known to acetylate p53 and function as coactivators, it will be interesting to determine whether simultaneous knockdown of p300 and CBP would still result in spontaneous p21 induction. Presumably, p53 should still exhibit the increased half-life in this setting that is seen in individual p300 or CBP knockdowns; however, if p300 or CBP-mediated

acetylation of p53 is necessary for activation of p21, as a recent report suggests (Tang et al., 2008), one might expect that stable p53 in this context may be unable to transactivate the p53 promoter.

In contrast, P/CAF knockdown had no significant effect on basal levels of p53 or p21 but, upon expression of p14ARF, completely prevented p53 stabilization and p21 induction, suggesting a critical role for P/CAF in p14ARF signaling (Figure 2.1). To ensure that these observations were not simply artifacts of the experimental system, the experiments were repeated in non-transformed RPE1 cells, which had been immortalized by the introduction of hTERT. Results from RPE1 cells closely matched those obtained from U2OS cells (Figure 2.1A-B), indicating that P/CAF plays a critical role in p53 stabilization and activation in response to ARF. Expecting that P/CAF was exerting its effects on p53 through acetylation, we examined post-translational modifications of p53 in response to p14ARF – specifically, S15 phosphorylation and K382 acetylation. Strikingly, although p14ARF expression stimulated both modifications, neither was detectable upon the depletion of any of the HATs tested (Figure 3.1 and 3.2). This suggests that the presence of all three HATs is required for S15 phosphorylation and K382 acetylation in response to p14ARF.

Two distinct possibilities arise from this observation: either that depletion of any one HAT makes the total pool of p53 HATs limiting for p53 acetylation, or that these HATs function together in concert or sequentially to direct p53 modification in response to p14ARF. These possibilities could be addressed by rescuing individual knockdowns by expression of heterologous HATs. This experiment also indicates that, in the case of p300

and CBP, these modifications are dispensable for activation of p21 expression. It will be important, however, to determine whether p21 induction by p300 or CBP knockdown is p53 dependent, and whether it is regulated at the level of transcription or protein stability.

Although no clear role for p300 and CBP in p14ARF signaling has been identified, some tangential evidence links the two – several reports have linked ATM to p14ARF function, while others have linked p300 to ATM function. The earliest report noted that S15 phosphorylation of p53 and cell-cycle inhibition directed by p14ARF are attenuated in cells from Ataxia Telangiectasia patients who harbor mutations in both ATM alleles (Li et al., 2004). A later report expands upon this observation, noting that p14ARF specifically activates ATM signaling in a p53-independent manner (Eymin et al., 2003). p300 activity has been linked to the DNA-damage response through roles in histone acetylation (Vempati et al., 2010), and through regulation of stability of NBS1, a factor critical to DNA-damage repair processes (Jang et al., 2011{Jang, 2010 #1079). This raises the possibility that p300 plays upstream roles in p14ARF-p53 signaling not limited to regulation of p53 stability, but involving ARF-directed activation of ATM signaling. It will be important to note whether ATM-signaling in this context does, in fact, impact p53 activation by ARF, and whether p300 regulates this process. The use of p300/CBP mutants deficient in E3 ligase or HAT activities to rescue knockdowns will be helpful in addressing these possibilities.

While it is clear that P/CAF regulates p53 levels and activity in response to p14ARF, how these effects are achieved is unclear. A recent report identified P/CAF as an E3 ligase for human MDM2 (Linares et al., 2007), an intriguing observation that could

potentially explain the role of P/CAF in p14ARF signaling; however, our repeated attempts to reproduce these results have so far failed, despite performing the experiments in the same cell type and using the same reagents. Specifically, where the authors see a marked elevation of MDM2 levels in response to P/CAF knockdown in U2OS cells after two hours, we observe no increase in MDM2 levels at this time point (Figure 2.7). The reasons for this discrepancy are unclear, but may relate to clonogenic differences between populations of the same cell type grown in culture, or may reflect a differential environmental stress between the two culture conditions. It is possible that specific conditions exist in which depletion of P/CAF would result in elevated MDM2 levels, but we have so far been unable to identify such a set of conditions.

Because ablation of P/CAF does not appear to alter basal levels of factors in the p14ARF-p53 response (Figure 2.1, Figure 3.3), we reasoned that P/CAF could be exerting its effects through modulation of protein-protein interactions or localization of a number of factors. To this end we examined the interaction of MDM2 with both p53 and p14ARF in p53-transfected H1299 cells (Figure 3.3). p14ARF and p53 co-immunoprecipitated efficiently with MDM2 in both the presence or absence of P/CAF, indicating that P/CAF is dispensable for both MDM2-p53 and MDM2-p14ARF interaction. We also examined the localization of MDM2 and p14ARF in this experimental setting, reasoning that perhaps P/CAF was necessary for p14ARF-directed nucleolar relocalization of MDM2. While significant background fluorescence of P/CAF staining was evident, MDM2 appeared to exhibit a greater degree of nucleolar exclusion in the absence of P/CAF (Figure 3.4).

This raises the intriguing possibility that a nucleoplasmic pool of active MDM2 exists which, upon p14ARF expression, is normally directed to nucleoli. In the absence of P/CAF, p14ARF may fail to relocalize MDM2 to nucleoli, leaving an active pool of MDM2 to antagonize p53 function even in the presence of ARF. Precisely how this would be achieved is not immediately clear – as mentioned above, P/CAF does not appear to impact the MDM2-p14ARF interaction; however, a more careful analysis should be performed to ensure that an MDM2-p14ARF complex is not reformed after lysis.

It is possible that P/CAF alters other, as yet undefined components of the MDM2-p14ARF complex. Gel filtration of the purified complex could aid in identification of new components of the complex, or at least provide qualitative data regarding the nature of the complex in the presence or absence of P/CAF. It is also possible that the novel E3 ligase activity of P/CAF could play a role in the regulatory ubiquitination of the p14ARF-MDM2 complex and facilitate its nucleolar translocation. While this is a preliminary result with which care should be taken to avoid over-interpretation, the possibility that P/CAF regulates MDM2 or p14ARF localization certainly warrants further attention. Attempts to rescue the defect in p53 stabilization with P/CAF mutants could also provide insight into which, if any, biochemical activities of P/CAF are necessary to promote ARF-induced p53 stabilization.

P/CAF: A Role in Tumor Suppression?

Dual critical roles for a coactivator in such a vital tumor suppressor pathway

might suggest that P/CAF itself is inactivated in some subset of tumors. The designation of a factor as an oncogene or tumor suppressor is, in many cases, straightforward; however, in the case of HATs, with a myriad of substrates often encompassing both oncogenes and tumor suppressors, the role of these factors in tumor progression is often highly context dependent. The impact of loss of activity of a HAT could presumably depend on the initiating lesion(s), the tissue affected, and the precise balance of oncogenes and tumor suppressors during the time at which a HAT becomes inactivated, whether through epigenetic means or loss of heterozygosity. It is reasonable to propose that if several driving oncogenes were dependent upon a HAT, its loss of activity might result in the elimination of the cell from the replicative pool; whereas, if wild-type p53 were present, loss of a critical HAT might phenocopy p53 loss and allow the cell to continue the progression toward a transformed state. If this in indeed the case, HATs in general should show a lower rate of mutation and exhibit a more limited spectrum of loss in human malignancies.

Evidence to date seems to support that loss of activity of the HATs discussed in this dissertation – p300, CBP, P/CAF, Tip60 – do, in specific cases, function as tumor suppressors and possibly (in rare cases) acquire oncogenic functions. Tip60, for example, commonly undergoes loss of a single allele in many lymphomas, mammary carcinomas, and head-and-neck carcinomas, defining Tip60 as a non-classical haploinsufficient tumor suppressor (Gorrini et al., 2007). CBP mutations cause the developmental disorder Rubenstein-Taybi syndrome, which is characterized by a high rate (about 5%) of neural crest tumors (Miller and Rubinstein, 1995). Many reports of spontaneous mutation of

p300 and CBP in human solid tumors have also been described. p300 is subject to inactivating mutations with LOH in gastric and colorectal tumors (Muraoka et al., 1996), and CBP in ovarian tumors (Ward et al., 2005). In most tumors screened, however, the rate of inactivating mutation with LOH was between 1-5% for p300 (Muraoka et al., 1996) (Gayther et al., 2000; Ozdag et al., 2002), and even more rare for CBP (Ozdag et al., 2002). Importantly, no significant inverse correlation appears to exist between p53 and CBP/p300 status, suggesting that the role of CBP/p300 inactivation in tumors is not confined to (and may be entirely independent from) those harboring wild-type p53. This suggests that, under certain conditions, p300 or CBP inactivation can contribute to tumorigenesis.

In leukemias and lymphomas, the role of p300 and CBP appear to be more complex, as they are, in rare cases, subjects of chromosomal rearrangements resulting in oncogenic fusion proteins (Borrow et al., 1996; Champagne et al., 2001; Giles et al., 1997). Despite this oncogenic gain-of-function, evidence from mice still suggest that loss of activity of p300 or CBP contribute to the onset of hematological malignancies, as blastocysts injected with p300 or CBP-null ES cells developed tumors (Rebel et al., 2002). Importantly, however, these experiments do not address a post-natal role for p300 and CBP in tumor suppression. It will be interesting to track future research regarding the mechanism through which p300 and CBP loss-of-function contribute to tumorigenesis.

Evidence for a role of P/CAF in tumor suppression has been more elusive. P/CAF-null mice develop normally and are not tumor-prone; however, it is important to note that GCN5 is heavily overexpressed in P/CAF-null mice, and likely functionally

compensates for P/CAF loss (Yamauchi et al., 2000). Furthermore, several early analyses failed to identify inactivating mutations of P/CAF at any significant rate in the tumors screened (Muraoka et al., 1996; Nishimori et al., 2000). Two recent reports, however, indicate that P/CAF inactivation contributes to tumorigenesis. A very recent report from the Avantaggiati lab (Perez et al., 2010) notes that P/CAF expression levels in a panel of colon, lung, head-and-neck, and bladder cancers are all significantly lower than matched non-tumor tissue. In another study, P/CAF activity appears to be lost through mutation or downregulation in a striking 60% of 80 esophageal squamous cell carcinomas screened. Reintroduction of P/CAF into cell lines established from several of these primary tumors potently inhibited tumorigenicity through reestablishment of a G1/S checkpoint associated with upregulation of, among other cell-cycle regulators, p21(Zhu et al., 2009). This raises the possibility that P/CAF does, in certain cases, function as a tumor suppressor through modulation of p53 activity by histone acetylation of target promoters. Because P/CAF does not appear to behave like a classical tumor suppressor, and is seemingly downregulated primarily by epigenetic means, a more thorough examination of P/CAF levels in various tumors may uncover a broader role for its involvement in tumor suppression.

Appendix I

The Transcriptional Adaptor hAda3 Interacts With, But Is Not Degraded By High-risk HPV E6

The hAda3 transcriptional adaptor, a component of the P/CAF HAT complex, has been identified as a necessary cofactor in p53 acetylation (Nag et al., 2007; Sekaric et al., 2007b)and has been identified as a target of high-risk E6-mediated degradation (Kumar et al., 2002). Degradation of hAda3 by E6 has been proposed to attenuate p53 activity in a manner independent from E6-mediated p53 degradation, and correlates well with the efficient immortalization of human mammary epithelial cells (MECs) (Shamanin et al., 2008), suggesting that degradation of hAda3 by E6 may represent an additional oncogenic function of high-risk E6 (of note, low-risk E6 is unable to promote degradation of hAda3).

The works cited above utilized expression of a FLAG-tagged hAda3 construct to demonstrate degradation by E6, due to the lack of antibodies recognizing native hAda3 at the time during which these experiments were performed. Generation of a polyclonal antibody in the Androphy lab generated by immunization with an N-terminal hAda3 peptide allowed us to study the impact of E6 on endogenous hAda3. We began by comparing the half-lives of untagged or FLAG-tagged hAda3 in H1299 cells expressing HPV 16E6 or a vector control by cycloheximide analysis. In agreement with recent reports, we observed a reduction in FLAG-hAda3 half-life in the presence of 16E6 (Figure A1.1A). Strikingly, however, we noted that the half-life of untagged hAda3 was

unaffected by 16E6 expression. Half-lives and steady-state expression levels of FLAG-tagged or untagged hAda3 were similar (Figure A1.1B).

Based on these results, we reasoned that native hAda3 may indeed interact with 16E6, and that the addition of FLAG tag may provide a lysine which could be targeted by the E6/E6AP complex. Alternatively, we thought it possible that the FLAG tag could induce a conformational change in hAda3 or itself provide an interaction site for 16E6. To address these possibilities, we performed co-immunoprecipitations of endogenous hAda3 in H1299 cells expressing 16E6 or a vector control. We noted a weak, but clean interaction between hAda3 and 16E6 (Figure A1.2), confirming that 16E6 and hAda3 do form a complex in cells.

That 16E6 does, in fact, not target hAda3 for degradation calls into question the physiologic role of such an interaction. Many possibilities exist – high-risk E6 could serve to inactivate several HAT complexes which are known to be limiting, through interaction with hAda3, thereby preventing coactivation of p53 or histone acetylation on p53-responsive promoters. Alternatively, it is possible that high-risk E6 itself utilizes the activity of HAT complexes to promote activation of factors such as c-myc or hTERT which contribute to E6-induced immortalization and transformation. A thorough analysis of the interaction of HAT complexes with their known transcription factors in the presence or absence of high-risk E6 will serve to address these questions.

Figure A1.1: hAda3 is not a target of 16E6-mediated degradation.

H1299 cells were plated at 5x10^5 cells per 60mm plate, and transfected the next day with 1ug hAda3 or FLAG-hAda3 (pCDNA3 vector backbone) and 1ug LXSN-16E6 or LXSN. 24 hours after transfection, cells were treated for the indicated durations with 50ug/mL cycloheximide (CHX). Cells were immediately lysed after CHX treatment in SDS buffer (2% SDS, 50mM Tris pH 6.8) **A)** 10ug lysates from each CHX treatment condition were separated by SDS-PAGE and blotted with the indicated antibodies (M5 anti-FLAG from Sigma-Aldrich, rabbit hAda3 antibody was generated and validated by the Androphy lab) **B)** 10ug of lysates from untreated samples (t=0) were separated by SDS-PAGE and blotted with the indicated antibodies (16E6 antibody was a generous gift from Arbor Vita Co.) to indicate steady-state levels of relevant factors.

Figure A1.1: hAda3 is not a target of 16E6-mediated degradation.

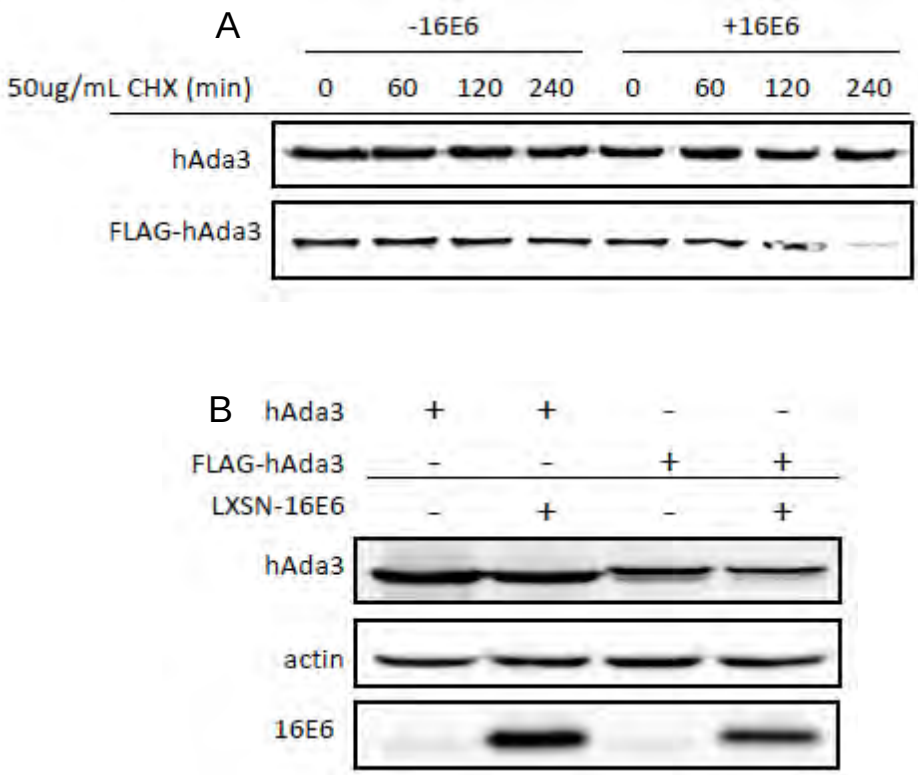
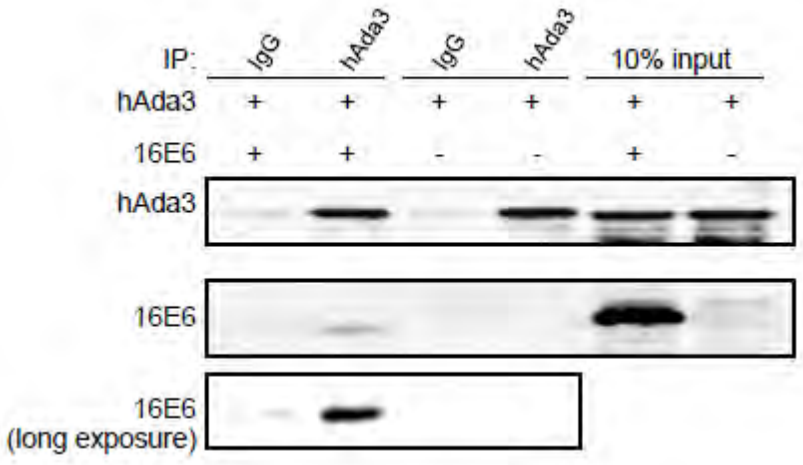


Figure A1.2: HPV 16E6 interacts with endogenous hAda3 in H1299 cells.

H1299 cells were plated at a density of 1.5x10^6 cells / 100mM dish and transfected as described in Figure A1.1. 24 hours after transfection, cells were lysed in 500uL IP-lysis buffer (50mM Tris pH 8.0, 100mM NaCl, 20mM NaF, 10mM KH2PO4, 1% Triton X-100, 2mM DTT, 10% glycerol, 1mM PMSF, 1x protease inhibitor cocktail (Roche)) for 30 minutes on ice. Immunoprecipitations were performed overnight in a total volume of 400uL using 200uL of the relevant lysate and final binding conditions of 100mM Tris pH 8.0, 100mM NaCl, 10mM NaF, 5mM KH2PO4, 0.5% Triton X-100, 2mM DTT, 5% glycerol, 1mM PMSF, and 1x protease inhibitor cocktail (Roche). The next day, 20uL PBS-washed protein A-agarose beads were added to each reaction and bound for an additional 2 hours. Reactions were washed 3 times each with 1mL was buffer (100mM Tris pH 8.0, 100mM NaCl, 0.5% NP-40, 1mM PMSF). Samples were eluted using 2x Laemmli buffer, boiled for 10 minutes, and separated by SDS-PAGE for analysis. Antibodies used for detection by western blot are described in Figure A1.1

Figure A1.2: HPV 16E6 interacts with endogenous hAda3 in H1299 cells.



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